Pest Control with Enhanced Environmental Safety

EDITED BY
Stephen O. Duke, Julius J. Menn,
and Jack R. Plimmer

ACS Symposium Series 524

ACS SYMPOSIUM SERIES 524

Pest Control with Enhanced Environmental Safety

Stephen O. Duke, EDITOR
Agricultural Research Service
U.S. Department of Agriculture

Julius J. Menn, EDITOR
Agricultural Research Service
U.S. Department of Agriculture

Jack R. Plimmer, EDITOR
ABC Laboratories

Developed from a symposium sponsored
by the Division of Agrochemicals
at the 203rd National Meeting
of the American Chemical Society,
San Francisco, California
April 5–10, 1992

American Chemical Society, Washington, DC 1993

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.fw001

Library of Congress Cataloging-in-Publication Data

Pest control with enhanced environmental safety / Stephen O. Duke, editor, Julius J. Menn, editor, Jack R. Plimmer, editor.

p. cm.—(ACS Symposium Series, ISSN 0097-6156; 524)

"Developed from a symposium sponsored by the Division of Agrochemicals at the 203rd National Meeting of the American Chemical Society, San Francisco, California, April 5–10, 1992."

Includes bibliographical references and index.

ISBN 0-8412-2638-5

1. Agricultural pests—Control—Congresses. 2. Agricultural pests—Control—Environmental aspects—Congresses. 3. Pesticides—Congresses. 4. Natural pesticides—Congresses.

I. Duke, Stephen O., 1944– . II. Menn, Julius J. III. Plimmer, Jack R., 1927– . IV. American Chemical Society. Division of Agrochemicals. V. American Chemical Society. Meeting (203rd: 1992: San Francisco, Calif.). VI. Series.

SB950.A2P467 1993
632'.9—dc20 93-12098
CIP

The paper used in this publication meets the minimum requirements of American National Standard for Information Sciences—Permanence of Paper for Printed Library Materials, ANSI Z39.48-1984. ♾

Copyright © 1993

American Chemical Society

All Rights Reserved. The appearance of the code at the bottom of the first page of each chapter in this volume indicates the copyright owner's consent that reprographic copies of the chapter may be made for personal or internal use or for the personal or internal use of specific clients. This consent is given on the condition, however, that the copier pay the stated per-copy fee through the Copyright Clearance Center, Inc., 27 Congress Street, Salem, MA 01970, for copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Law. This consent does not extend to copying or transmission by any means—graphic or electronic—for any other purpose, such as for general distribution, for advertising or promotional purposes, for creating a new collective work, for resale, or for information storage and retrieval systems. The copying fee for each chapter is indicated in the code at the bottom of the first page of the chapter.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

American Chemical
Society Library
1155 16th St. N. W.
Washington, D. C. 20036

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.fw001

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.fw001

1993 Advisory Board

ACS Symposium Series

M. Joan Comstock, *Series Editor*

V. Dean Adams
Tennessee Technological University

Robert J. Alaimo
Procter & Gamble Pharmaceuticals, Inc.

Mark Arnold
University of Iowa

David Baker
University of Tennessee

Arindam Bose
Pfizer Central Research

Robert F. Brady, Jr.
Naval Research Laboratory

Margaret A. Cavanaugh
National Science Foundation

Dennis W. Hess
Lehigh University

Hiroshi Ito
IBM Almaden Research Center

Madeleine M. Joullie
University of Pennsylvania

Gretchen S. Kohl
Dow-Corning Corporation

Bonnie Lawlor
Institute for Scientific Information

Douglas R. Lloyd
The University of Texas at Austin

Robert McGorrin
Kraft General Foods

Julius J. Menn
Plant Sciences Institute, U.S. Department of Agriculture

Vincent Pecoraro
University of Michigan

Marshall Phillips
Delmont Laboratories

George W. Roberts
North Carolina State University

A. Truman Schwartz
Macalaster College

John R. Shapley
University of Illinois at Urbana–Champaign

L. Somasundaram
E. I. du Pont de Nemours and Company

Peter Willett
University of Sheffield (England)

In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.fw001

Foreword

THE ACS SYMPOSIUM SERIES was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of this series is to publish comprehensive books developed from symposia, which are usually "snapshots in time" of the current research being done on a topic, plus some review material on the topic. For this reason, it is necessary that the papers be published as quickly as possible.

Before a symposium-based book is put under contract, the proposed table of contents is reviewed for appropriateness to the topic and for comprehensiveness of the collection. Some papers are excluded at this point, and others are added to round out the scope of the volume. In addition, a draft of each paper is peer-reviewed prior to final acceptance or rejection. This anonymous review process is supervised by the organizer(s) of the symposium, who become the editor(s) of the book. The authors then revise their papers according to the recommendations of both the reviewers and the editors, prepare camera-ready copy, and submit the final papers to the editors, who check that all necessary revisions have been made.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

M. Joan Comstock
Series Editor

In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.

Preface

RECENT ADVANCES IN THE DISCOVERY and development of new synthetic and natural pest management agents and technologies that might play a role in reducing the environmental impact of pest control are the focus of this volume. The material is organized into sections on weed, insect, and plant pathogen management. Within each section are chapters on synthetic compounds, biotechnology, and natural products.

An introductory chapter sets the stage for the book, summarizing the current state of pest control and the increased pressures to develop safer and more environmentally benign pesticides and pest control technologies. Weed management tools are addressed in eight chapters. An overview of biorational design of herbicides is provided in the first chapter of this section. Glufosinate is an environmentally and toxicologically benign herbicide, and a chapter is dedicated to the production of crops that are resistant to this herbicide. The next chapter discusses synergism as a strategy for reduction of herbicide use rates. This is followed by consideration of the potential of the porphyrin pathway as a target site for herbicide action.

Three chapters on various aspects of biocontrol of weeds with mycoherbicides are presented. The first outlines the technological barriers of this approach, and the subsequent chapters offer potential solutions in overcoming some of these barriers. The final chapter of the weed management section considers the potential of natural products as sources of new, safer herbicides.

The section on insect control is addressed in eleven chapters and covers a broad cross section of the latest technologies involving chemical compounds and semiochemicals in insect management. The material covered includes chapters on the application of insect receptor technology to discovery of selective insecticides, novel insect growth regulators, new insecticides based on heterocyclic moieties, natural products as insecticides, viral and microbial insecticides and use of semiochemicals in managing parasitoids, and a final chapter on host plant resistance to insect attack based on genetically engineered cotton plants expressing an insecticidal protein toxin.

A final section deals with management of plant pathogens. Chapters on naturally occurring nematicides, microbial compounds to control plant diseases, biocontrol of plant pathogens, and safer fungicides are found in this section.

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.pr001

Not every important pesticide, biocontrol agent, or biotechnological approach to pest control is covered in these 24 chapters. However, our hope is that this eclectic collection of chapters on chemical strategies and technologies will provide an accurate view of where we are and where we are going in pest management.

This volume is the outgrowth of a symposium held under the auspices of the Agrochemicals Division, American Chemical Society, at the national meeting in April 1992 in San Francisco, California. The symposium spanned two complete days, with half-day sessions in the areas of herbicides, insecticides, biopesticides, and natural products and semiochemicals.

We thank the contributors to this volume for outstanding efforts in synthesizing the latest information on their topics. We also acknowledge the time and expertise of the many reviewers who helped to make these chapters even better. The assistance of Anne Wilson, Maureen Rouhi, Barbara Tansill, Cheryl Shanks, Donna Lucas, and other staff at the Books Department of the American Chemical Society is gratefully acknowledged. Finally, we thank the Agrochemicals Division of the American Chemical Society and the Agricultural Research Service of the U.S. Department of Agriculture for their willingness to support our symposium.

STEPHEN O. DUKE
Agricultural Research Service
U.S. Department of Agriculture
Stoneville, MS 38776

JULIUS J. MENN
Agricultural Research Service
U.S. Department of Agriculture
Beltsville, MD 20705

JACK R. PLIMMER
ABC Laboratories
Columbia, MO 65205

October 9, 1992

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.pr001

Chapter 1

Challenges of Pest Control with Enhanced Toxicological and Environmental Safety

An Overview

Stephen O. Duke[1], Julius J. Menn[2], and Jack R. Plimmer[3]

[1]Southern Weed Science Laboratory, Agricultural Research Service,
U.S. Department of Agriculture, P.O. Box 350, Stoneville, MS 38776
[2]Plant Sciences Institute, Beltsville Agricultural Research Center,
Agricultural Research Service, U.S. Department of Agriculture,
Beltsville, MD 20705
[3]ABC Laboratories, 7200 ABC Lane, Columbia, MO 62505

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

Much of the increase in agricultural productivity over the past half century has been due to more efficacious and economical pest control through the use of synthetic chemical pesticides (SCPs). However, there is continued and growing social and legislative pressure to reduce the toxicological and environmental risks associated with control of agricultural pests with SCPs. Public and private sector research is being conducted to develop birational pesticides and to replace or reduce the use of SCPs with natural product-based pesticides, biocontrol (including classical biocontrol), genetically-engineered pest resistance, and combinations of these replacement strategies. Nevertheless, these emerging pest control technologies will likely represent only a small percentage of the pest control market by the year 2000. Therefore, methods to reduce use rates of synthetic pesticides and to develop more environmentally and toxicologically benign pesticides are also important in risk abatement. Such strategies as birational design, development of pesticide synergists, and development of crops resistant to more environmentally safe herbicides, insects, and plant pathogens can improve the environmental quality, food safety, and allay societal fears concerning crop protection technology.

In the 1990's, crop protection chemicals continue to be the major tool for protecting food and fiber crops from damaging insects, weeds and plant pathogens. The 1990 end-user world market value of pesticide chemicals reached almost $23 billion (*1*). Herbicides accounted for a 47% share, insecticides accounted for 28%, and fungicides for a 22% share of the world market. According to an unpublished study by McKinsey and Company, the current market share for biologicals was less than 1% in 1990. However, this sector will probably grow by 10% per annum through the remainder of the 1990's. Most likely, this growth will emanate from nonrecombinant bioproducts, including various strains of *Bacillus thuringiensis* (*Bt*), bioherbicides and biofungicides (*2*). Even with a 10% growth rate per year, biologicals will only represent about 2 to 10% of the pest control market by the turn of the century.

0097–6156/93/0524–0001$06.00/0

The growth of biological pesticides will largely benefit from growing constraints imposed on synthetic chemical pesticides, including the high cost of development and registration, real and perceived food safety issues, environmental concerns, resistance of pests to pesticides, and social perceptions of risk associated with chemicals.

Further impetus for the development of biologicals is coming from increased attention to and application of integrated pest management (IPM) practices in the agriculturally developed countries.

It is likely that, in the next 5-10 years, the private sector will endeavor to develop more selective, efficacious, biodegradable, and nonhazardous chemical pesticides. These advances will be stimulated by greater emphasis on biochemical design, pharmacology, molecular design and synthetic modifications of natural products. In the public sector, research will concentrate on unconventional crop protection techniques; primarily on technologies that are highly compatible with IPM biologically based pesticides including microbials, viruses, semiochemicals, host plant resistance, and classical biological control agents. In the long term, recombinant DNA technology is likely to have a significant impact on pest management through recombinant microbials and transgenic crops.

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

Environmental Considerations and Public Policy

Growth of Pesticide Use and Environmental Concerns. The history of chemical pest control has been largely a record of success. The introduction of this technology worldwide has benefited the lives of millions of humans by improving health and nutrition. Campaigns against disease vectors have increased life expectancy in many tropical countries. In India in 1953 there were 75 million cases of malaria. By 1962, 147 million pounds of DDT had been used, so that by 1967 there were fewer than 100,000 cases (*3*). Synthetic chemicals have been remarkably effective in protecting crops against insects and diseases and in reducing the dependence of the farmer on laborious hand weeding. An outcome of the changes brought about in farming is that the food supply of the United States is now produced by only 5% of the population (*4*). These early successes were so spectacular that they obscured a fuller understanding of the implications of pesticide use. Its widespread adoption was assured but there was a general failure to take into account the environmental impact of pesticide use and to examine its more subtle ecological effects.

As part of an expanding chemical industry, pesticide manufacture developed rapidly after the second World War. Conditions favored the marketing of pest control chemicals. There were substantial incentives for the farmer to increase yields. Food prices rose and, as more affluent populations demanded more food of higher quality, farmers in the developed nations of the world prospered. The chemical manufacturers sought new markets, and the promise of DDT and other new synthetic insecticides showed that chemical control of pests had rich potential as a new area for market development.

There were excellent opportunities for commercial successes and, because the biological activities of wide chemical classes of synthetic organic compounds had been largely unexplored, random screening programs offered a good chance of success within a short span of time. Therefore, the industry grew remarkably between 1945 and 1975 when the world output of pesticides increased from 100 to 1,800 thousand tons (*5*).

This growth appeared to be limited only by the further development of market and the ability of the chemist to synthesize new molecules possessing activity which differed sufficiently from those produced by industrial competitors to warrant patent protection. However, constraints were soon imposed on rapid growth by findings that pointed out some negative aspects of SCP use. Not only were some pesticides extremely resistant to degradation, but it was also found that among large pest populations, some individuals survived pesticide treatment to become the progenitors of populations that were unaffected by subsequent use of the pesticide.

Initially, justification for widespread pesticide application was provided by the belief that organic compounds would be ultimately degraded by soil organisms to mineral fractions (carbonate, nitrate, chloride, etc.). When developments in analytical instrumentation during the 1950s made available rapid, economical, and sensitive techniques for measurement of pesticide residues in food and environmental samples, it became possible to obtain realistic measurements of their occurrence and distribution. Although soil had been thought to possess an almost infinite capacity for mineralization, measurements of levels of organochlorine insecticides showed that the process could be extremely slow and that residues could also be transported from their site of application to become widely distributed in the environment.

The chemical stability of organochlorine pesticide compounds and their lipophilicity were properties that favored their bioaccumulation. Residues in wildlife became widely distributed and, as their effects on birds, fish, and other species were studied, it became clear that the survival of some species was threatened and that some human and animal food items were at risk of pesticide contamination.

Clearly, the continued use of such pesticides was undesirable and it was apparent that there had been insufficient study of the implications of pesticide use. However, it was felt that if there was adequate information about the environmental fate of pesticides and their effects on biota the issue of harm to the environment could be resolved by improvements in technology. The key to the solution of the problems was thought to lie in designing suitably modified chemical structures that had little or none of the undesirable properties of the persistent pesticides.

This approach was followed with considerable success. Such compounds as the pyrethroid insecticides demonstrated the ingenuity of the chemist in combining pesticidal activity with low mammalian toxicity and extremely low potential for environmental harm. However, as understanding of environmental effects grew, the criteria by which environmental damage was judged also expanded to include such factors as the potential to contaminate water sources or to pose a potential threat to endangered or beneficial species. An extremely difficult question is that of determining the absence or presence of subtle effects in natural ecosystems. The search for answers to these questions and technological solutions was not undertaken until pressures from society developed. A combination of societal initiatives and legislative actions have placed substantial constraints on the development of new SCPs.

Regulation and Public Policy. The environmental movement that found expression in such works as *Silent Spring* (*6*) acquired sufficient strength and political support to affect legislation during the 1970's. In December 1970, the U.S. Environmental Protection Agency (EPA) was formed and took over many pesticide regulatory functions that were formerly the responsibility of the U.S. Department of Agriculture. Manufacturers seeking registration of pesticides began to receive requests for environmental fate data, and in 1975 the detailed guidelines for registration introduced by the EPA brought registrants face to face with comprehensive questions of safety and environmental persistence.

The Federal Insecticide Fungicide and Rodenticide Act (FIFRA) has evolved through several versions since its first enactment in 1947 (*7*) and under FIFRA the EPA has authority to register or approve pesticide uses in the U.S.A. The basis

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

of EPA's approval is an assessment of the benefits of the pesticide use against the potential risks to human health and the environment. Under the Federal Food, Drug and Cosmetic (FFDC) Act, the EPA is charged with setting tolerances for pesticide residues that can be present in or on foods sold in interstate commerce. This process was examined recently in a National Research Council Report (*8*) commissioned by the EPA which asked the Board on Agriculture of the National Research Council to study the EPA's tolerance setting process, particularly with regard to current and future impacts of the Delaney Clause. This clause is a proviso of the FFDC Act that prohibits the setting of a tolerance for any pesticide that concentrates in processed foods and has been found to induce cancer in animals.

Under FIFRA, the manufacturer or registrant submits data to EPA, including chemical and toxicological properties, possible effects on wildlife, plants and other biota in the environment, and likely distribution in the environment. In addition, before pesticide uses are approved for food crops, a tolerance must be granted under section 408 of the FFDC Act for residues in raw agricultural commodities and under section 409 of the FFDC Act for pesticides that concentrate in food.

The process of tolerance-setting requires much of the same information as is required by the EPA to support registration. The registrant must furnish information on the quantities of residues expected to be present in meat, milk, poultry, fish, and eggs derived by feeding pesticide-treated products to animals. Toxicity tests are required for the parent compound, major impurities, degradation products, and metabolites.

The studies required include both acute and chronic testing. Tolerances set by EPA for non-oncogenic pesticides are based on assessment of the risk faced by the individual consumer, derived by a calculation that requires knowledge of the "no observable effect levels" and assumptions concerning the dietary contribution of each food item that might contain residues of the pesticide. For oncogenic pesticides, a different process must be used to determine whether the use of the pesticide presents an undue risk to humans.

When FIFRA was enacted in 1947, it included a provision for keeping registration data current and a 5-year renewal requirement was introduced. This was used primarily as a method for removing products that were no longer of interest to registrants. In 1988, amendments to FIFRA were enacted on the procedures for conduct of the reregistration process. This process has a lengthy history and the issue of reregistration has been discussed by Gray (*9*). Although reregistration was envisaged as a part of the registration process, it was not until the 1988 amendments to FIFRA were approved that resources became available to make re-registration effective.

The amendment of FIFRA in 1964 brought about changes that included cancellation of pesticides that posed unacceptable risks. The procedural rights of the registrant included referral to an advisory committee and an adjudicatory hearing, if the cancellation process was initiated. USDA regulations extended registrants formal hearing rights to disputes over 5 year renewals. Thus, the concept of registration developed almost as a property-like right to market a product. The concept that this was an almost permanent right created a favorable climate for industrial investment in pesticide research and development.

In 1972, FIFRA was replaced by the Federal Environmental Pest Control Act (FEPCA) which modified the provisions of FIFRA by further regulating the use of pesticides by introducing the classifications of 'restricted use and general use.' The former were to be used only by trained, certified applicators. The law also called for reregistration and classification of all products marketed between 1974 and 1976.

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

By making it illegal to use a pesticide in a manner inconsistent with the label, the act recognized the principle that the risk of adverse effects was dependent on the way in which a toxic substance was handled. The provisions of this act also affected the evaluation of pesticides and addressed the need to reduce risks to humans and the environment. This is being achieved by processes that evolve continuously within federal and state agencies.

Environmental chemistry study requirements for pesticides are discussed in Subdivision N of the EPA guidelines for Environmental Fate Studies (40 CFR Subsection N Sections 161-165). These call for the following types of studies:

Hydrolysis
Aerobic and anaerobic metabolism in soil
Soil leaching and adsorption
Laboratory and field volatility
Dissipation studies
Accumulation studies

Some of these studies are conducted in greenhouse or laboratory situations and interpretation of data is straightforward. However, some field studies may require more complex and costly experimental designs and the data may be difficult to interpret. The necessity to conduct water monitoring studies is an example of such a study. Because large-scale studies may cost several hundred thousand dollars, there is reluctance to conduct such investigations until they are deemed essential by regulatory agencies. The implementation of such a study also delays the completion of the dossier that must be submitted by the registrant, and thus the entry of the chemical into particular markets is also delayed.

There is now a wealth of data concerning the environmental behavior of pesticides and it has become clear that several problems that currently attract attention could not be foreseen on the basis of existing knowledge and data acquired at a time when scientific understanding of environmental fate was more limited. As an example, the widespread contamination of water by trace quantities of herbicides was not perceived as a potential problem until herbicides had been used continually over large acreages for an extended period.

Variability between and within geographical sites is a major problem in the interpretation and design of field experiments and data assessment. Such difficulties may hinder agreement between registrant and regulator as to the design of studies that will provide satisfactory data for environmental assessment. In some cases, models may be useful for understanding and predicting environmental behavior. However, their ability to predict subtle effects may be limited. Unpredicted, but measurable, contamination may result from a very small fraction of applied pesticide.

The need to meet requirements for data concerning environmental aspects of pesticide use places a considerable burden on registrants. The data needs of the regulatory agencies must be constantly modified to accommodate new scientific findings, and the continuing nature of the process is formalized by re-registration. The recognition that criteria for acceptance are likely to undergo continuous modification has challenged the pesticide industry to seek novel approaches to molecular design and has stimulated the development of alternative pest management strategies.

In 1978, EPA separated the classification process from re-registration, and amendments to FIFRA in that year provided EPA with authority to require submission of additional data concerning registered products.

The October 1978 FIFRA provisions provided a schedule for completion of data submission and review. Fees paid by registrants provided resources to accelerate

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

completion of the re-evaluation by EPA. The re-registration process must be completed by 1997. It has compelled industry to discontinue many products and uses because the cost of re-registration did not justify the expenditure or diversion of resources.

The impact on agrochemical development has been substantial. In 1988, it was stated that a total of $70 million may be expended to develop and market a new pesticide before there is a positive cash flow from sales, and profitability may not be realized until the 20th year after discovery (*10, 11*). The amount which must be spent to obtain data concerning environmental behavior has increased considerably as the regulations continue to embrace more aspects of environmental behavior. Public opinion increasingly presses for new legal measures to reduce human exposure to pesiticides, and attention is being drawn to all potential routes by which humans might be exposed. These concerns extend to beneficial insects and to nontarget organisms. Concern for nontarget organisms may conceivably generate a climate that favors long-term ecological studies as an obligatory part of the safety tests. Such tests may greatly prolong the period before a pesticide may reach the market.

Since patent rights expire 17 years after being granted, incentives to develop and register new pesticides in the United States have been considerably reduced.

Aspects of Newer Approaches to Pest Control

Efficacy and Reliability. One of the chief reasons for the continued use of SCPs is that they have been generally more efficacious, expeditious, and reliable than alternative methods of pest control. Pest control methods that are ineffective or unreliable will not be used. Within limits, the economic value of a pest control agent or method increases with efficacy and reliability. Lack of reliability and/or efficacy has prevented the adoption of many pest control agents (biological and chemical) and methods, even though they appear more toxicologically and environmentally acceptable than currently used synthetic pesticides.

For example, numerous indigenous fungi have been patented for biocontrol of weeds (*12-14*). These products have the potential to replace certain synthetic herbicides. Several have undergone some level of development by private industry. However, only two have actually been brought to the marketplace in the United States, and these products have very limited markets. A primary reason for the limited development of these biocontrol products is insufficient efficacy and relatively high cost. Further research on formulation, strain selection or manipulation, application technology, and storage could eventually make more of these products competitive with synthetic pesticides. Much of this research may have to be conducted by the public sector because there is little profit incentive for many potential biocontrol products, especially those that propagate, spread, and persist (*15*).

In the non-recombinant DNA area, significant strides have been made with semiochemicals and biocontrol agents in the management of insect pests. Notable advances have been made with improved strains of *Bt*, fermentation products such as the avermectins and milbemycins, semiochemicals (*16*) and natural products such as azadirachtin derived from the neem tree (*17*).

Current developmental efforts include work with improved baculoviruses, engineered baculoviruses and transgenic plant species such as cotton expressing the *Bt* toxin. It will take several more years to determine the efficacy and market acceptance of these innovative selective and environmentally safer insect control technologies.

Specificity and Selectivity. One of the first concerns with a new pesticide or pest control measure is that it should be specific for the target organism(s). An overriding consideration is that it is not significantly toxic to humans, livestock, or pets. Furthermore, the more non-target species to which the product or method is benign, the higher its likelihood of success. However, extreme selectivity can limit or prevent the commercial success of a pesticide or pest control measure unless the target species is a very important one, and its control has the potential for significant economic returns.

Most SCPs were discovered empirically in industrial laboratories. These chemicals were primarily broad spectrum insecticides, such as the chlorinated hydrocarbon insecticides, carbamates, organophosphorus ester insecticides (OP's), and synthetic pyrethroids (SP's). As already mentioned, current and future discovery efforts will be based more heavily on: 1) natural product models, 2) biorational design based on biochemical principles, and 3) quantitative structure activity relationships (QSAR) analyses of selective chemistries (*18, 19*).

The newer approaches to discovery of insecticides with selective toxicity to insects and mites have resulted in diverse new chemical moieties and structures. These include even newer synthetic pyrethroids, benzoyl- phenylureas, novel heterocyclics, fermentation derived products such as the avermectins and milbemycins, neem-derived pesticides, and novel insect growth regulators having juvenoid action (*20*).

To date, the ultimate in specificity and selectivity in insect management has been achieved with semiochemicals. This technology has had mixed success in the past, but it is currently expanding to employ semiochemicals, especially sex pheromones, in mating disruption and mass trapping programs (*16*).

Weed problems generally involve several weed species. The ideal herbicide is highly phytotoxic to a broad range of weeds, with no phytotoxicity to non-target plant species. Most commercial synthetic herbicides only approach this ideal, possessing limited phytotoxicity to the crop at application concentrations that do not completely eradicate the weeds. The principal reason for this is that crops are tolerant to most herbicides due to metabolic degradation rather than by resistance at the molecular site of action. Several new methods offer improvements in crop tolerance. Genetically engineered crops can be made completely resistant to herbicides by alteration of the site of action. For example, the broad spectrum herbicide glyphosate that will kill most plant species at economical rates can be used on transgenic crops with an altered site of action (*21*). Some biological control agents are highly selective for particular weeds, with no effect on certain crops (*13, 14*). In many cases, they are too selective to justify their cost, especially when the target weed is not a major weed problem. However, plant pathogens with a broad range of hosts might be genetically manipulated to reduce their host ranges or survival capacity without an obligate inoculum formulation (*22*).

Just as with synthetic herbicides, the phytotoxicity of naturally-occurring compounds ranges from non-selective to very selective. In fact, certain microbial phytotoxins (host-specific phytotoxins) that are generated by plant pathogens are more selective than any synthetic herbicides, affecting only one species or a variety of a species (*23*). Only one such compound (the cyclic dipeptide, maculosin) has been found to be host-specific for a weed species (spotted knapweed) (*24*). As with the biocontrol agents, the economic viability of an extremely selective phytotoxin is questionable unless the target pest causes great economic loss.

A better understanding of weed and crop biochemistry has the potential for helping in the biorational design of herbicides with more desirable patterns of selectivity. A biochemical site can be chosen which is unique to plants in order to minimize mammalian toxicity. Furthermore, a site can be chosen which differs

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

significantly between a crop and certain weeds associated with it. To date, this process has acted in reverse. That is, in studying the mechanisms of tolerance and selectivity to synthetic herbicides discovered by non-biorational methods, we have discovered underlying biochemical and physiological differences between weeds and crops.

Toxicity. The impetus for new pest control measures is fueled in large part by a public concern that currently used pesticides are undesirable or unacceptable from a toxicological standpoint (*e.g.*, *25*). The health risk of currently used synthetic pesticides is a matter of public and scientific debate, although the natural toxicants found in our food supply may cause considerably more human health problems than pesticide residues (*26*). Regardless of the outcome of this debate, there will be continued emphasis on development of pest control products and methods that minimize toxicological risks.

Quantitative as well as qualitative aspects of toxicological risk must be evaluated. For example, improved formulations (*e.g.*, with synergists or encapsulation) or methods of application technology could reduce the efficacious use levels of a previously undesirable compound to levels that might pose no health risk.

All new products and methods for pest control, including non-synthetic pesticides, should be carefully evaluated for possible toxicological hazards. For example, the toxicological risks of altering plants by breeding or biotechnology to enhance levels of natural pesticidal toxicants should be as rigorously evaluated as synthetic pesticides and their metabolites. Transgenic crop plants with genes from unrelated organisms should be examined for their biosynthesis of unexpected toxicants (*27*).

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

Soil and Water. Contamination of groundwater and surface waters with pesticides has become a major public issue. Reported cases of groundwater contamination are increasing rapidly. In part, this is due to reductions in levels that are detectable because of improved analytical capabilities. It also results from decades of heavy pesticide use in many areas. Once a groundwater supply is contaminated with xenobiotics, there is a need for risk analysis to determine if potential human or animal health problems exist and to estimate the cost of contaminant removal before use.

In recent years, contamination of groundwater and surface water with the insecticide/nematicide aldicarb and certian soil applied and/or incorporated herbicides has been of greatest concern. Most insecticides are applied as foliar applications to crops; however, 20-25 million acres of corn are treated annually with OP and carbamate insecticides incorporated into soil for control of the corn rootworm complex (CRC) (*2*). Due to these massive applications to soil, concerns have arisen about potential groundwater and/or surface water contamination. These concerns have accelerated research into alternative strategies to manage the CRC that would reduce dependence on prophylactic treatment with soil applications of insecticides. These tactics involve the combination of semiochemicals with minute amounts of an insecticide in a bait formulation applied to control the above ground adult stage of the CRC. These approaches are providing new opportunities for development of specific attractants, feeding arrestants, non-polluting formulations, and delivery systems (*2*).

Herbicides are used in larger amounts than other pesticides (*28*), and many of them are applied directly to soil or incorporated into soil. Certain herbicides and their metabolites will move readily to groundwater in some soil types. Contamination of groundwater and surface waters with triazine and chloroacetamide (*e.g.*, alachlor, metolachlor) herbicides has been widely reported (*29-32*). There is controversy over how much of this contamination is due to point sources (e.g., spills, improper disposal of containers, etc.) versus normal use.

This has caused industry to shift its emphasis from soil-applied and preplant-incorporated herbicides to postemergence, foliar-applied herbicides. Furthermore, considerable effort has been made to reduce point source contamination. For example, some companies now sell their products in reusable, returnable, containers to be used in fully-contained spray systems (*32*).

A shift from soil-incorporated and soil-applied herbicides to postemergence, foliar-applied herbicides would decrease the potential for groundwater contamination. Most newer herbicides being developed are designed for postemergence, foliar application. Also, crops are being genetically engineered to be resistant to non-selective herbicides that are ideal for postemergence weed control. However, increased use of post emergence herbicides could lead to increased surface water contamination with herbicides.

Conservation of soil and water is an important aspect of agricultural land management. Conventional weed control requires tillage, primarily because the herbicides available will not adequately control weeds without tillage. The array of new, more powerful and selective herbicides available for some crops (*e.g.*, soybeans) have made reduced or no-tillage methods feasible. This method conserves both soil and soil moisture. Furthermore, it eventually reduces the reservoir of weed seed buried in the soil. Biotechnological generation of crops resistant to more environmentally benign, broad-spectrum herbicides (*e.g.*, glyphosate or glufosinate) should allow more wide spread adoption of no-till farming, while reducing the toxicological and environmental impact of herbicides.

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

Pest Resistance

Pest resistance to pesticides is a natural process that develops and spreads in response to selection pressure. Without intervention, resistance will, at some point, limit the efficacy of most crop protection chemicals, including primarily insecticides, fungicides, and acaricides. To date, development of resistance to insecticides has been documented in over 500 species of insects and mites (*33*). In several species, resistance to multiple chemical insecticides has been detected, further exacerbating the problem. Critical cases of resistance have been encountered in at least a dozen or more major economic pests including the Colorado potato beetle, sweetpotato whitefly and the diamondback moth (*33*). No class of insecticides has escaped the resistance syndrome, including the biological insecticide *Bt* (*33*).

The development of significant levels of resistance to pesticides will adversely affect agriculture and society at large. Diminished efficacy of useful pesticides may lead to significant increases in costs of pest management due to increased rates and/or frequency of pesticide applications and the need to change to more costly crop protection tactics. Among the more promising elements of a strategy to counter pesticide resistance are intensified efforts in early monitoring of resistance, implementation of biologically based control measures, less frequent pesticide applications, and alternating types of chemical pesticides.

Development and spread of resistance of weeds to herbicides has been slower than that of insecticide resistance because of several factors. These include: 1) the much longer generation time of weeds, 2) the lengthy dormancy period of seeds of many species, 3) the limited mobility of weeds, 4) the often less constant and severe selection pressure of herbicides during a generation than that of insecticides, and 5) the fact that herbicides with several different mechanisms of action are generally used over ten or twenty weed generations (only one insecticide

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

or chemical class is often used over the same number of insect generations). Nevertheless, over the past 15 years, herbicide resistance has grown from a rare occurrence to a common one (*28, 34*). For example, resistance to triazine herbicides was first reported only about 25 years ago. It has now been detected in more than 60 species, representing more than 40 genera. Resistance to many other herbicides with entirely different molecular sites of action has been reported. Resistance to several important new classes of herbicides (sulfonylureas, imidazolinones, and others) can appear in only three or four years of use, apparently due to considerable genetic diversity and/or plasticity in the gene(s) that codes for the enzymic site of action (acetolactate synthase)(*35*). Cases of multiple resistance (that is, resistance to several unrelated herbicide classes) are being reported and have become very difficult problems in isolated cases (*36, 37*).

These factors have led to forecasts of greatly increased problems with herbicide resistance during the next decade. Availability of fewer herbicide alternatives, as the reregistration process takes its toll, may exacerbate these problems. The flow of new herbicides with new sites of action into the market has diminished to a trickle due to the increasingly high cost of herbicide registration. Furthermore, in most cases, it is unlikely that alternative methods of weed control will soon provide effective and economical alternatives to the chemical methods of the past four decades. All of these factors suggest that resistance of weeds to herbicides will become a very costly problem in the near future. Effective strategies, such as crop and herbicide rotation, to delay or prevent the appearance of herbicide resistance should be formulated and utilized (*38*).

Impact of Biotechnological Methods on Pest Management

New and developing biotechnology has the potential to dramatically affect all aspects of how humankind interacts with its biological environment, including pests. Recombinant genetic methods allow alteration of both the biological targets of pests, such as crops, or the natural enemies of pests, for more effective biological control.

Industry sources project that in the next decade, the year 2000, and beyond, the market value of biopesticides and biotechnology products useful in crop protection may reach $6 to $8 million per annum (*2*). These high values, while speculative, are predicated on significant breakthroughs in the development and market acceptance of highly potent microbial, viral and fungal control agents through recombinant DNA technology and successful introduction of transgenic plants expressing engineered insect toxins such as the *Bt* endotoxin and resistance to plant pathogens.

Many new avenues of non-conventional chemical pest controls are currently opening. It is likely that successful IPM in crop protection will be based on the best elements of judiciously applied selective chemical control agents in combination with biocontrol products and technologies.

For insect and pathogen control, crops can be genetically altered to produce insecticides and/or fungicides. Existing genetics of the plant can be manipulated to increase levels of natural pesticides, or foreign genes can be introduced to provide the biosynthetic machinery to generate new natural pesticides. The most publicized visible example of this is that of *Bt* toxins.

It is also feasible to engineer crops to generate their own herbicides; however, the crop has to be resistant to the herbicide and it must be a compound that would be leached from the roots in sufficient quantity to effectively kill or inhibit growth of competing weeds. Presently, there is little effort to produce crops that are more allelopathic by biotechnology or conventional breeding. Many plant breeders are of the opinion that high levels of allelopathic compounds were bred out of crops centuries ago in order to make them more palatable and productive. Furthermore, there is little evidence that allelopathy plays a critical role in most plant-plant interactions in nature.

The generation of crops resistant to existing herbicides is a more straightforward method for use of biotechnology in weed control. Numerous crops have been made resistant to a variety of herbicides via several different biotechnological methods (*21, 39, 40*). Concern has been expressed that the use of biotechnology to generate herbicide-resistant crops will increase and perpetuate the use of herbicides, ultimately leading to unnecessary toxicological and environmental problems (*41-43*). However, this technology can be used to generate crops resistant to more environmentally safe herbicides than are currently used on those crops. In some cases, several herbicides or a herbicide plus a synthetic herbicide safener can be replaced with one herbicide through use of herbicide-resistant crops. Furthermore, preemergence herbicides, used as insurance before the extent of a weed problem is known, can be replaced with postemergence herbicides that will be used only when and where a significant weed problem exists. Used judiciously, herbicide-resistant crops can also be valuable in strategies to prevent occurrence and spread of herbicide-resistant weeds. However, some have warned that herbicide-resistant crops themselves could become weeds or transmit resistance genes to weed species (*39, 44*). Nevetheless, all crops are tolerant to some herbicides and, to date, there are no reported cases of herbicide resistance arising from interspecies gene transfer or of crops becoming weeds simply because they are tolerant to certain herbicides.

Biotechnology can also be used in the biocontrol of weeds. There is little present interest in utilization of biotechnology to generate insects for control of weeds. Research into weed control with insects is almost exclusively devoted to the classical approach of introducing a new insect to a weed population to which the weed has no host defense. However, many biotechnological aspects of weed control with microbes are being explored. Most of this effort is associated with use of inundative treatment of weeds with endemic pathogens (*13, 14*). This technology has not been very successful because of limited host-ranges of many of the pathogens, inadequate virulence, and extremely variable results with different environmental conditions. Manipulation of virulence, host-range, and reliability of these weed control agents by formulation, strain selection, and genetic modification is being studied to make this technology competitive with chemical weed control methods. For example, Sands *et al.* (*22*) have proposed to genetically engineer pathogens with broad host ranges so that they are dependent on a component of their formulation for infectivity.

Genetic modification of endemic plant pathogens to be used for biocontrol is not without risk. Certain genetic alterations could result in novel host ranges that might include crops, or the altered pathogen might transfer foreign genes to related microbes, to produce unwanted new pathogens.

Future Directions of Pest Management

The relative roles of current and developing methods of pest control in the future will depend on a myriad of factors. These include the rising political, environmental, and economic costs of managing pests almost entirely with synthetic chemical pesticides. These costs will be determined by the stringency of registration and re-registration requirements, as they are subjected to the influences of scientific judgement, public opinion, and political pressure. The costs of discovery and production of improved pesticides in a replacement market

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

are rising, while discovery efforts are meeting diminishing returns. The efficacy and economics of new, alternative methods of pest control with real or perceived environmental advantages over older methods will critically affect the equation. Improved and more powerful technology, including biotechnology, has the potential of revolutionizing pest control. However, these newer methods are not without environmental, toxicological, and economic risks. Each new product, strategy, or method will have to be weighed against alternatives from several perspectives. As the human population outpaces food production, methods of pest control that maximize production might eventually be favored over those that minimize toxicological or environmental risks, even though this seems unlikely in the present political climate. Nevertheless, many of the potential future directions of pest control that are perceived as environmentally and toxicologically safer than present methods will probably be favored in the developed world.

Literature Cited

1. Anon. *Agrow World Crop Protection News*, **1991**, *135*, 18.
2. Menn, J.J., *Amer. J. of Indus. Med.* **1990**, *18*, 499-504.
3. Anon. *Agriculture - Environmental and Consumer Protection Appropriations for 1974: Hearings before a Subcommittee of the Committee on Appropriations, House of Representatives, 93rd Congress,* U.S. Govt. Printing Office, 1973, p. 645.
4. see ref. 3, p. 637.
5. Green, M.B., Hartley, G.S., West, T.F. *Chemicals for Crop Improvement and Pest Management*, Pergamon, Oxford 3rd Edition 1977, page 33.
6. Carson, R. *Silent Spring*, Houghton Mifflin, Boston 1962
7. Anon. *Federal Insecticide, Fungicide and Rodenticide Act* enacted by Public Law 92-516, U.S. Code, Title 7, Part 136 et seq., October 21, 1972.
8. Anon. *Regulating Pesticides in Food, The Delaney Paradox, Board on Agriculture,* National Research Council, National Academy Press, Washington, DC, 1987.
9. Gray, E.C. In *Regulation of Agrochemicals*; Marco, G.J., Hollingworth, R.M., Plimmer, J.R., Eds.; Amer. Chem. Soc., Washington, D.C., 1991; pp. 45-54.
10. Leng, M.L. In *Regulations of Agrochemicals*; Marco, G., Hollingworth, R.M., Plimmer, J.R., Eds.; Amer. Chem. Soc., Washington, D.C., 1991; pp. 27-44.
11. Menn, J. J.; Henrick, C. A. In *Agricultural Chemicals of the Future*, J. L. Hilton, Ed.; Rowman and Allanheld, 1984; pp. 247-265.
12. Ayres, P.; Paul, N. *New Scientist*, **1990**, *127* (1), 36-39.
13. Charudattan, R. *Amer. Chem. Soc. Symp. Ser.* **1990**, *439*, 132-154.
14. Templeton, G. E.; Heiny, D. K. In *New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases*, R.R. Baker and P. Dunn, Eds.; Alan R. Liss, Inc., 1990; pp 279-286.
15. Crawley, M.J. *Brighton Crop Cont. Conf. - Weeds* **1989**, *3*, 969-978.
16. Menn, J.J. *Crop Protection,* **1991**, **10**, *347-353.*
17. *Schmutterer, H. J. Insect Physiol.* **1988**, *34*, 713-719.
18. Morrod, R.S. *Phil. Trans. R. Soc. London.* **1981** *295*, 35-44.
19. Magee, P.S.; Kuhn, G.K.; Menn, J.J. *Amer. Chem. Soc. Symp. Ser.* **1985** *225*, 1-207, ACS, Washington, DC.
20. Henrick, C.A. *Proc. Conf. Insect Chem. Ecol. Ta'bor*, **1990**. 429-452. Academia Prague and SPB Acad. Publ. The Hague.
21. Duke, S. O.; Holt, J. S.; Hess, F. D.; Christy, A. L. *Herbicide-Resistant Crops,* Comments from CAST, no. 1991-1; Council for Agricultural Science and Technology, Ames, IA, 1991, 24 pp.
22. Sands, D. C.; Miller, R. V.; Ford, E. J. *Amer. Chem. Soc. Symp. Ser.* **1990.**, *439*, 184-190.

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

23. Scheffer, R. P.; Livingston, R. S. *Science* **1984** *112*, 17-21.
24. Stierle, A.; Cardellina, J.H.; Strobel, G. *Proc. Natl. Acad. Sci. USA* **1989,** *85*, 8008-8011.
25. Huff, J. E.; Haseman, J. K. *Chem. Eng. News* **1990,** *69*, 33-37.
26. Ames, B. N.; Profet, M.; Gold, L. S. *Proc. Natl. Acad. Sci. USA.* **1990,** *87*, 7777-7781.
27. International Food Biotechnology Council. *Regul. Toxicol. Pharmacol.* **1990,** *12*, i-S196.
28. LeBaron, H. M.; McFarland, J. *Amer. Chem. Soc. Symp. Ser.* **1990,** *421,* 336-352.
29. Belluck, D.A.; Benjamin, S.L.; Dawson, T. *Amer. Chem. Soc. Symp. Ser.* **1991,** *459*, 254-273.
30. Cohen, S. Z., Eiden, C.; Lorber, M. N. *Amer. Chem. Soc. Symp. Ser.* **1986,** *315*, 170-196.
31. Pereira, W. E.; Rostad, C. E. *Environ. Sci. Technol.* **1990,** *24*, 1400-1406.
32. Moll, S. *Brit. Crop Protect. Conf. - Weeds - 1991,* **1991,** *2*, 703-707.
33. Georghiou, G.P. *Amer. Chem. Soc. Symp. Ser.* **1990,** *421*, 18-41.
34. Holt, J. S.; LeBaron, H. M. *Weed Technol.* **1990,** *4*, 141-149.
35. Saari, L. L.; Cotterman, J. C.; Primiani, M. M. *Plant Physiol.* **1990,** *93*, 55-61.
36. Holtum, J.A.M., Powles, S.B. *Brit. Crop Protect. Conf. - Weeds -1991,* **1991,** *3*, 1071-1078.
37. Powles, S.B.; Matthews, J.M. In *Resistance 91: Achievments and Developments in Combating Pesticide Resistance.* Elsevier Press. - In Press.
38. Gressel, J.; Segel, L. A. *Amer. Chem. Soc. Symp. Ser.* **1990,** *421*, 430-458.
39. Quinn, J. P. *Biotech. Adv.* **1990,** *8*, 321-333.
40. Mazur, B. J.; Falco, S. C. *Annu. Rev. Plant Physiol. Mol. Biol.* **1989,** *40*, 441-470.
41. Goldburg, R.; Rissler, J.; Shand, H.; Hassebrook, C. *Biotechnology's Bitter Harvest.* Biotechnology Working Group. 1990, 73 pp.
42. Miller, M. *J. Environ. Health* **1991** *54*, 13-14.
43. Hindmarsh, R. *The Ecologist* **1991,** *21*, 196-205.
44. Williamson, M. In *Herbicide Resistance in Weeds and Crops*. Butterworth-Heinemann Ltd., Oxford, 1991; pp. 375-386.

RECEIVED July 31, 1992

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch001

Chapter 2

Target-Site Directed Herbicide Design

L. M. Abell[1], J. V. Schloss[2], and A. R. Rendina[1]

[1]Agricultural Products, Stine-Haskell Research Center, E. I. du Pont de Nemours and Company, Newark, DE 19714
[2]Department of Medicinal Chemistry, University of Kansas School of Pharmacy, Lawrence, KS 66045

The successful design of novel pesticides depends on careful consideration of a number of factors including target enzyme selection and validation, inhibitor design, delivery of the inhibitor to the target, and its metabolic fate. Strategies for the selection of enzyme targets giving the desired physiological response upon partial inhibition include identification of chemical leads, bacterial and plant conditionally lethal mutants and the use of antisense technology. Enzyme inhibitors having pharmaceutical or agrochemical utility can be categorized into six major groups: ground-state analogues, group specific reagents, affinity labels, suicide substrates, reaction intermediate analogues, and extraneous site inhibitors. Examples of each category, and their advantages and disadvantages, will be discussed. Identification of a target and construction of a potent inhibitor in itself may still not lead to an effective herbicide. In the absence of the desired in vivo activity, the uptake, translocation, and metabolism of the inhibitor should be studied to assess the full potential of the target. Strategies for delivery of the compound to the target enzyme and avoidance of premature detoxification may include a proherbicidal approach, especially when inhibitors are highly charged or when selective detoxification or activation can be exploited. Utilization of differences in detoxification or activation between weeds and crops may lead to enhanced selectivity. Without a full appreciation of each of these facets of pesticide design, the chances for success with the target or enzyme-driven approach are reduced.

In our experience the target-site directed approach to herbicide design requires the close interaction of an interdisciplinary team composed of plant physiologists, biochemists, enzymologists, molecular biologists, and synthesis chemists. The approach can be divided into three main processes; initial selection of a suitable target enzyme, design and synthesis of a potent inhibitor using detailed knowledge of the enzyme reaction and kinetic mechanism, and optimization of the lead structure into an effective herbicide by translation of *in vitro* activity of the inhibitor into *in vivo* efficacy. This last step may or may not be required, depending on the observed effects of the inhibitor on the whole plant and the extent to which consideration of

0097–6156/93/0524–0016$06.50/0

other limiting factors such as uptake, translocation, and detoxification were given in the design process. In this paper we will focus on the design of enzyme inhibitors using examples applicable to crop protection chemistry whenever possible. Brief discussions of potential strategies for target selection and validation as well as for optimizing efficacy of potent inhibitors are also included.

Many of the examples that follow are from studies on established herbicide targets with inhibitors that were discovered empirically. In many cases, especially those involving active site directed inhibitors, we believe that modern inhibitor design strategies could have produced the same compounds that were discovered by screening methods. Furthermore, we are convinced that these types of studies can be applied to novel targets and will ultimately lead to the discovery of new herbicides through target-site directed programs in the near future .

Target Selection - Choosing the Right Enzyme

Perhaps the most obvious choice of a target for inhibitor design are enzymes already identified as targets for commercial herbicides or ones that are recognized as targets for naturally occuring phytotoxins (for a summary of known molecular modes of action see Duke et al. (*1*) and references therein). One very large advantage to this approach is that new inhibitors are virtually guaranteed to produce a herbicidal effect provided that the compounds reach their target. Extremely potent, second generation inhibitors have been designed for a number of known herbicidal targets. However, this increased potency does not always translate into improved *in vivo* activity. Inhibitors that are more potent than the commercial herbicides have been designed for the following known targets: acetolactate synthase (ALS) (*2*), acetyl-CoA carboxylase (ACC) (Rendina, A.R.; Hagenah, J.A.; Wollenberg, R.H.; Cherpeck, R.E., Chevron Chemical Company, unpublished data, Rendina, A.R; Taylor, W.S., E.I. Du Pont de Nemours, unpublished data); 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase (*3*), and glutamine synthetase (GS) (*4-8*). However, none have shown greater activity or more desirable selectivity than the commercial products. In the first three cases, the designed inhibitors clearly had unfavorable physical properties, such as too many negative charges at physiological pH or a higher overall lipophilicity, that would lead to poor uptake and/or translocation in the plant. New inhibitors of established targets may also experience weed resistance problems brought about by the original chemicals, especially if the new inhibitors occupy a similar binding site. While successful exploitation of a known target site has not yet yielded a new commercial product using the inhibitor design approach, continued study of these known targets promises to be successful ultimately.

Clearly, while having a chemical lead with known phytotoxic effects and known target enzyme is the safest way to choose a target, the choice is limited to a few enzymes and has the potential drawbacks discussed above. Application of modern molecular biological techniques may reduce the time needed for elucidation of sites of action for other classes of empirically discovered herbicides and plant pathogen phytotoxins. Once uncovered, these new "chemically validated" target sites can then be further exploited using mechanistic information to design better enzyme inhibitors.

Target Selection Criteria. In the absence of a chemical lead with a known site of action, the following idealized criteria may be used to guide the selection of a suitable target. First, the target enzyme should catalyze a reaction that is essential to the plant and/or inhibition of the targeted enzyme should lead to multiple deleterious effects. There is increasing evidence that simply introducing a block in the production of an essential component in the plant may not yield phytotoxic effects that are comparable to those produced by known herbicides (*9, 10*), but may simply produce a static or growth inhibition effect. Ideally, inhibition of the target should deprive the plant of an essential component and either deplete the plant of essential metabolic intermediates, consume high energy intermediates in a futile cycle, cause a toxic intermediate to accumulate, or any combination of the above. Second, the target enzyme should be present in plants, but not mammals in order to minimize the potential for undesirable toxicity. General toxicity of chemicals, however, may have little to do with inhibition of the chosen target and is very difficult to anticipate. For example, the commercialized inhibitors of GS and ACC have very low mammalian toxicity despite the presence of these enzymes in mammalian tissues. Third, the intracellular concentration of the target should be low in order to increase the likelihood for low use rate herbicides with reduced environmental consequences. For example, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) would make a poor target because of the high abundance of target molecules (Rubisco is a relatively slow catalyst and C3 plants contain up to one-half of the leaf-soluble protein as Rubisco). Therefore, even with an ideal inhibitor that titrates the target enzyme *in vivo*, requiring only one inhibitor molecule per molecule of enzyme, the large number of Rubisco molecules would set the lower limit on the use rate. (In tobacco leaves the concentration of Rubisco active sites is approximately 100 μM, which would correspond to a use rate of about 50 kg/ha for an ideal inhibitor with a molecular weight of 250 g/mole.) A fourth criterion is that the target enzyme should be practical from inhibitor design, synthesis, and delivery standpoints. Some enzyme mechanisms are inherently unsuitable for inhibitor design, kinases being one example where simple acid-base chemistry is used to effect phosphoryl transfer from a relatively complex cosubstrate (ATP), and these enzymes should be avoided. Also, inhibitors based on mechanistic studies of the target enzyme should be practical from a synthetic standpoint. Potential synthetic problems may be anticipated by avoiding enzymes with extremely complex substrates or intermediates. Likewise, target molecules that are highly charged or too lipophilic for good uptake and translocation should be avoided. Finally, from a practical standpoint, it is desirable that the target enzyme can be readily assayed, preferably from plant tissue. For mechanism-based inhibitors it should be possible to use a bacterial source for the target enzyme provided the catalytic mechanisms do not vary between bacteria and plants.

Target Validation (or Invalidation)

To help justify the resources required by target-site directed research, it is very important to know whether inhibition of the chosen target is sufficiently lethal to produce potent herbicidal effects. When only modest inhibition of the target is achieved with little or no whole plant activity, a decision whether to obtain the needed mechanistic information to design more potent inhibitors must be made. Additional effort is more likely to be expended if there is strong evidence that inhibition of the target enzyme is lethal to the plant. Using the criteria described in the previous section to select the target enzyme will help reduce the number of targets and provide some confidence that inhibition will lead to herbicidal effects. But are there ways to predict which enzyme in a biosynthetic pathway is the most lethal target? The approaches used to address this question can be divided into chemical and genetic methods.

Chemical Validation. Currently most target-site design work involves "chemical " validation of the target. That is, potent and selective inhibitors of the target enzyme are designed, synthesized, and tested both *in vitro* and *in vivo*. In many cases, good *in vitro* activity has been observed with moderate, little, or no effect on the whole plant level. In most of these cases it is not clear whether these compounds are weak herbicides due to an intrinsically poor target site or if uptake, translocation, or metabolic degradation is reducing herbicidal activity. If weak *in vivo* activity is observed with a potent inhibitor of the target enzyme, the next step should be to investigate the uptake, translocation, and metabolism of the radiolabeled inhibitor to be sure that the compound reaches the target enzyme without being detoxified. In addition, to ensure selectivity of the compound for the chosen target enzyme, specific enzyme activity levels relative to uninhibited controls can be determined. This information is most easily obtained with tight-binding or irreversible inhibitors. Alternatively, decreases in end products and accumulation of substrates or substrate precursors can be monitored. Reversal experiments with biosynthetic intermediates that are downstream from the inhibited site can also verify that the inhibitor is truly affecting the intended target. If the obvious reasons for lack of efficacy of the inhibitor (uptake, translocation, detoxification, and inhibition) cannot explain its lower *in vivo* potency, then it would seem that inhibition of this target enzyme in plants is intrinsically less phytotoxic than inhibition of known herbicide target sites. This enzyme may need to be inhibited to a greater extent, and/or for a longer period of time than can be achieved, to result in plant death. Another possible explanation for the lower herbicidal effect is the presence of an alternate enzyme activity that is insensitive to the inhibitor. To help define what constitutes a lethal target site, additional studies are needed on the known enzyme targets to elucidate the sequence of events that ultimately lead to death. Such studies would help establish criteria for testing unproven target sites.

Each individual enzyme in an essential metabolic pathway should be validated separately rather than assuming that all are equally valid targets. For example, in the branched chain amino acid biosynthetic pathway, where the first enzyme, ALS, is the known target for several classes of herbicides (*11, 79*), inhibitors have been designed for the second and third enzymes in the pathway, ketol-acid reductoisomerase (KARI)(*12, 13*) and dihydroxy acid dehydratase (*14-16*), respectively. Although the inhibitors were not of equal potency, only inhibitors of KARI showed any herbicidal activity (weak to moderate). In the aromatic amino acid biosynthetic pathway, where EPSP synthase is the established target for the herbicide, glyphosate (*17*), inhibitors have been prepared for dehydroquinate (DHQ) synthase (*18-20*), dehydroquinase (Rendina, A.R.; Lawyer, A.L.; Cherpeck, R.E.; Wollenberg, R.H., Chevron Chemical Company, unpublished data) (*21*), chorismate synthase (*22-23*), and chorismate mutase (*24-26*). Again, despite the fact that not all of the inhibitors were of equal *in vitro* potency, only inhibitors of DHQ synthase and chorismate synthase produced herbicidal effects (modest). However, none of the inhibitors of these alternate targets in either pathway have yielded whole plant effects that approach the potency achieved with the inhibitors of targets of the commercial herbicides. The reasons for this lack of *in vivo* potency has yet, for the most part, to be elucidated.

In only one case, KARI, have all the appropriate physiological studies been conducted to demonstrate clearly that the enzyme was a poor target (*27*). In conjunction with detailed mechanistic work, Aulabaugh et al. (*12*) were able to design and prepare potent inhibitors of KARI. Despite being better inhibitors of KARI than the most potent herbicidal inhibitors of ALS, the oxalyl hydroxamates were only weak to moderate herbicides. Additional studies with radiolabeled inhibitor showed that uptake, translocation, and metabolism were not significant factors in reducing the effectiveness of these compounds as herbicides in susceptible plants. Because of the extreme potency of the oxalyl hydroxamate inhibitors and their slow binding properties, residual KARI activity could be measured *in vivo* at different inhibitor levels. At levels of inhibition greater than 90% *in vivo*, no cytotoxic effects (such as reduction in cell volume) were observed. Only when KARI was inhibited 95-98% was an accumulation of acetoin observed along with a concommitant reduction in cell volume. These results suggest that KARI is simply not as good a target enzyme as ALS because the plant has considerably more enzyme than it needs to survive. Similar results were obtained with HOE 704, a phosphine oxide inhibitor of KARI, discovered via screening rather than by design (*13*).

Genetic Validation. From these examples we have learned that not all enzymes in a pathway are equally good targets. It may be more expedient to validate potential targets using genetic approaches. In the genetic approaches to target validation, the methods are designed to detect which enzyme in a metabolic pathway will give rise to lethal effects when inhibited and to estimate how much inhibition is required to produce those effects. Approaches used thus far involve studying plant mutants to determine what altered gene products result in lethal phenotypes, using bacterial systems as models to detect deleterious effects of blocking individual steps in a biosynthetic pathway (*10*), and using antisense genetic constructs to vary the level of expressed activity of specific enzymes.

In the first approach, higher plant mutants are obtained and the mutant phenotype is determined under conditions of starvation for the endproduct of the pathway or other stringent conditions. This approach is limited by the availability of suitable mutants having different levels of expression of the targeted enzyme. In most cases the mutants contain a complete block in the pathway of interest and serve only to validate the entire pathway rather than an individual component (*30-32*). A good example of the potential utility of this approach comes from a careful study of mutants in barley having different levels of expression of chloroplastic glutamine synthetase (*33, 34*). Glutamine synthetase plays a central role in the assimilation of ammonia in plants and, more importantly, in the recycling and recovery of amino donors and carbon lost during photorespiration. Plants which contain mutations in GS and other photorespiratory enzymes are isolated at high levels of CO_2 where photorespiration is suppressed. These mutant plants are air-sensitive when exposed to light, but survive as non-mutant phenotypes in the dark under normal atmospheric conditions. Mutant plants that contained less than 40% of the wild type level of GS activity began to accumulate ammonia and showed reduced rates of CO_2 fixation under normal photorespiratory conditions (ambient levels of CO_2 and O_2). These results suggest that GS would be a good target for herbicide design since only partial blockage would result in phytotoxic symptoms. This enzyme is a known target for an empirically discovered commercial herbicide, glufosinate, which contains the natural product, phosphinothricin, as the active ingredient (*35, 36*), so it has been validated both genetically and chemically.

Another genetic approach which is being increasingly used in plant biochemistry and has the greatest promise for providing quantitative results is to use antisense constructs of the targeted gene to determine the level of inhibition required for lethality. In this context, antisense refers to production of messenger RNA (mRNA) from the opposite strand of the DNA than that normally used for biosynthesis of a protein, thereby reducing the expression of that protein in most cases. The antisense approach involves cloning the cDNA or gene for the target from a plant species, chosing a promoter for the antisense construct (usually a strong one that produces an expression pattern similar to the native gene), making antisense contructs, transforming and regenerating plants, and checking the phenotypes of transformants to determine whether they correlate with reduction in activity of the enzyme target, or the level of its mRNA or protein. The expression level of the target is influenced by the number of copies of antisense genes introduced and the relative strengths of the promoters for the native and antisense genes.

In plants antisense RNA has been used effectively to reduce the activity of nopaline synthase (*37,38*), chloramphenicol acetyltransferase (*39,40*), chalcone synthase (*41,42*), polygalacturonase (*43,44*), phosphinothricin acetyltransferase (*45*), β-glucuronidase (*46, 47*), granule-bound starch synthase (*48*), Rubisco (*49, 50*), 1-aminocyclopropane-1-carboxylate synthase (*51*) and oxidase (*28, 52*), and phenylalanine ammonia-lyase (PAL) (*53*). Although these antisense experiments were not conducted to validate these enzymes as potential herbicide targets, we can interpret the results from that perspective. With the exception of Rubisco, greater than 90% reduction of activity compared to wild type levels was needed in each case before noticeable symptoms or effects on the whole plant could be observed. Significant growth reduction was observed in transgenic tobacco where Rubisco levels were reduced by 82% with antisense RNA (*49*); however, Rubisco is not a good herbicide target site due to its high concentration in plants (see earlier discussion). These results suggest that none of these enzymes are good targets for herbicide design, and demonstrates the utility of the method in eliminating potential targets.

To illustrate the above method in more detail, the results obtained with phenylalanine ammonia-lyase can be examined. This enzyme is a potential herbicide target because of its importance in the biosynthesis of lignin. Antisense constructs in tobacco plants reduced PAL activity by 88% in leaves and 95% in petals after 11 weeks without severely impairing the growth of the genetically altered plants compared to the wild type. The transgenic plants did have unusual phenotypes such as localized fluorescent lesions, altered leaf shape and texture, stunted growth, and altered flower morphology and pigmentation, but none of these effects were lethal (*53*). It is interesting that almost exactly the same phenotypes were observed when plants were treated with potent PAL inhibitors such as aminooxy-β-phenylpropionic acid, which is bound 30,000 times more tightly than the substrate (*54, 55*), again without severe phytotoxic consequences. Thus, both chemical and genetic approaches have effectively invalidated this enzyme as a target for herbicide design.

The use of antisense constructs to validate targets has the potential for being quite effective since targets which are attractive from an inhibitor design standpoint can be chosen for antisense studies to determine the level of inhibition required for phytotoxicity. If phytotoxic effects are observed, but the level of inhibition required is high (>95%, which is higher than can easily be achieved with an inhibitor), then the enzyme may be eliminated as a target. In addition, the antisense approach is potentially more efficient than chemical validation and will be increasingly applied in the crop protection industry. The approach has limited utility if the gene of interest codes for a very lethal site (obtaining transformants and regeneration may be difficult especially at high levels of inhibition), if there are multiple (highly divergent) genes for the same target site (redundant copies of the gene exist, but only one is affected by the antisense construct), if other mutations are induced by the transformation procedure that obscure the phenotypic effects of the antisense mutation, or if antisense does not work for that particular gene (transformation-induced mutations are present, but little or no reduction in enzyme activity is observed).

Enzyme Inhibitor Design

Enzyme inhibitors having agrochemical utility can be categorized into six major categories: group specific reagents, ground-state analogues, affinity labels, suicide substrates, reaction intermediate analogues, and extraneous site inhibitors. A number of reviews on this topic are available which can be consulted for more examples and for an analysis of the kinetic characteristics of each type of inhibitor (*63, 72*).

Group-specific Reagents. Group-specific reagents are intrinsically reactive toward the types of functional groups normally found in proteins. Protein functional groups which can be modified include cysteine sulfhydryl groups, the ε-amino groups of lysine, the carboxylate group of aspartate or glutamate, the phenolic hydroxyl of tyrosine, the thio ether linkage of methionine, and the indole moiety of tryptophan. The amino and carboxyl termini of proteins may also be modified readily. Group-specific reagents generally exhibit very little specificity for a single enzyme as protein reactive groups are ubiquitous. These reagents are, therefore, used almost exclusively as a research tool to identify residues in the binding domain of substrates or cofactors and to correlate structure with function rather than to design selective inhibitors. One example of a group of successful herbicides which might be classified as group specific reagents are the chloroacetamide herbicides (Figure 1). These herbicides contain a reactive halogen which is a common feature of many known protein modification reagents, one example being iodoacetamide. The halogen is potentially quite susceptible to displacement by nucleophilic groups in either proteins or metabolites, and these herbicides are known to alkylate coenzyme A and a number of proteins *in vivo* (*56*). In addition, a correlation has been found between the herbicidal

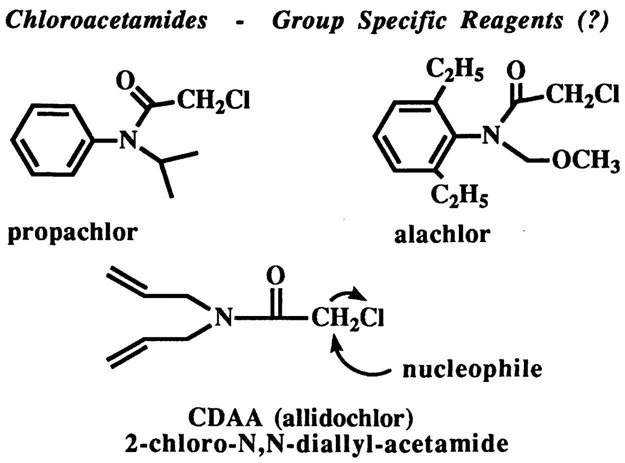


Figure 1. Examples of chloroacetamide herbicides - possible group specific reagents.

activity of chloroacetamides and their electrophilicity (*57*). Consistent with the reactivity of these herbicides, the primary mode of action has not been identified, although they are known to interfer with a number of processes including lipid metabolism (*56*), protein, anthocyanin, and gibberellin biosynthesis. One hypothesis for the mode of action of these herbicides is that their potency is derived from the combined effects of multiple sites of action (*58*).

Ground State Analogues. Ground state analogues of substrates, cofactors, or allosteric effectors may also be used as enzyme inhibitors. The use of substrate analogs can be quite selective for a single enzyme, providing the substrate is unique to that enzyme. Two such substrate analogues are methyl-(4 aminophenyl sulphonyl) carbamate (the commercial herbicide, asulam) and 6-fluoro-5-enolpyruvylshikimate-3-phosphate (6-fluoro-EPSP) (Figure 2).

The antibacterial and herbicidal activity of asulam requires conversion *in vivo* to *p*-aminobenzenesulfonamide (sulfanilamide) which in turn inhibits folate biosynthesis (*59, 60*). This compound is a competitive inhibitor for dihydropteroate synthetase and has a K_i of 5.7 μM compared to the K_m of 0.57 μM for the normal substrate, *p*-aminobenzoic acid (*59*). Sulfanilamide was also found to be an alternate substrate for the enzyme. However, it is the inhibition of dihydropteroate synthetase which is responsible for the antibacterial activity of this compound (Figure 2, top). Inhibition of other enzymes by the product of sulfanilamide and 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine is inconsequential.

While asulam was discovered through an empirical approach, 6-fluoro-EPSP was designed as a result of mechanistic studies on the enzyme chorismate synthase, the seventh enzyme in the shikimate pathway. Chorismate synthase catalyzes the conversion of EPSP to chorismate (Figure 2, bottom). While the chemical mechanism for the conversion of EPSP to chorismate is not entirely elucidated, studies have shown that the pro-R hydrogen is abstracted and that there is a primary kinetic isotope effect at C-6 on V_{max}, indicating that proton abstraction is at least partially rate limiting (*61*). (6R)- and 6S)-6-fluoro-EPSP were synthesized and found to be competitive inhibitors of chorismate synthase with respect to EPSP with K_i's of 3 μM and 0.2 μM, respectively, compared to the K_m for EPSP of 2.2 μM (*23*). While 6-fluoro-EPSP is itself unsuitable as a herbicide, mostly for uptake reasons, a proherbicide approach may be used in this case. Two enzymatic transformations were used in the synthesis of 6-fluoro-EPSP. 6-Fluoro-shikimate was converted to 6-fluoro-shikimate-3-phosphate using shikimate kinase and then converted to 6-fluoro-EPSP

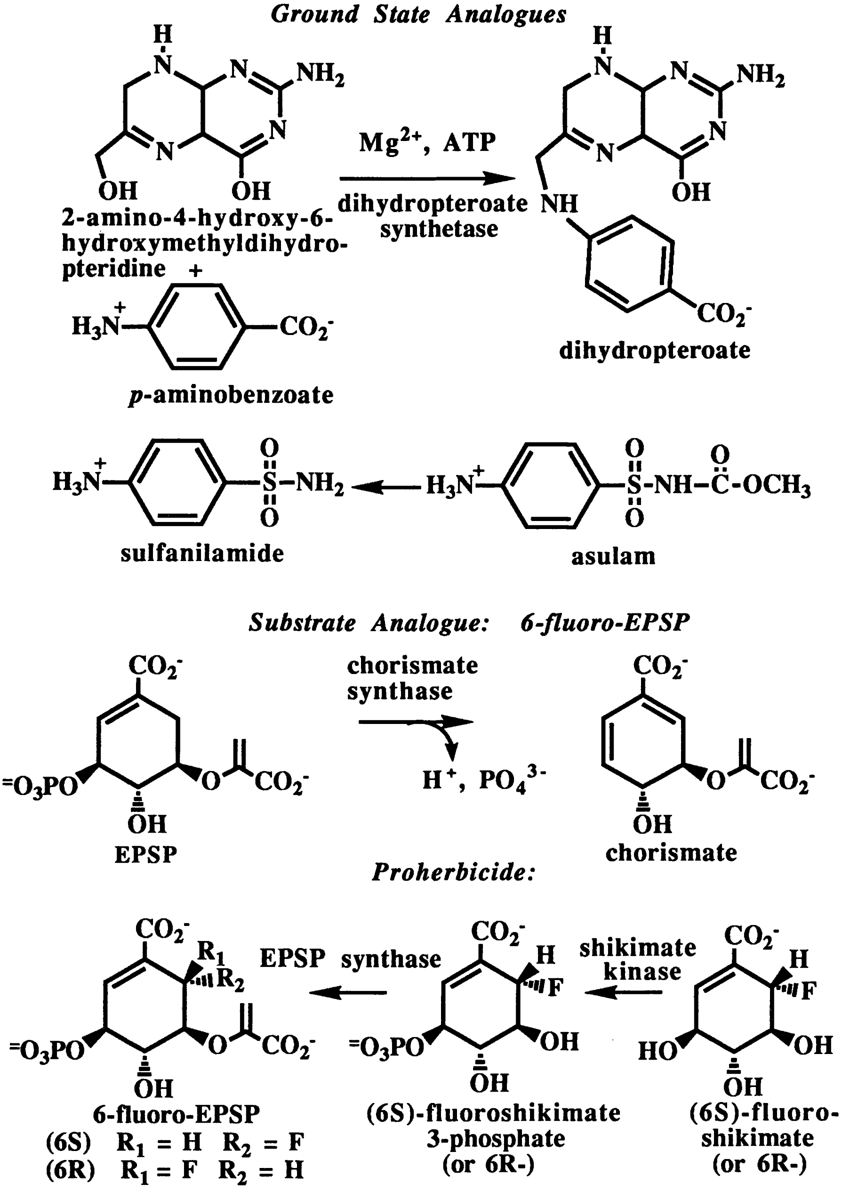

Ground State Analogues
2-amino-4-hydroxy-6-hydroxymethyldihydropteridine +
p-aminobenzoate
Mg2+, ATP
dihydropteroate synthetase
dihydropteroate
sulfanilamide
asulam
Substrate Analogue: 6-fluoro-EPSP
EPSP
chorismate synthase
H+, PO43-
chorismate
Proherbicide:
EPSP synthase
shikimate kinase
6-fluoro-EPSP
(6S) R1 = H R2 = F
(6R) R1 = F R2 = H
(6S)-fluoroshikimate 3-phosphate (or 6R-)
(6S)-fluoro-shikimate (or 6R-)

Figure 2. Examples of ground state analogue inhibitors for dihydropteroate synthetase (top) and chorismate synthase (bottom).

using EPSP synthase. The later step is an order of magnitude slower with the fluorinated substrate compared to the normal substrate (*23*). These results indicate that 6-fluoro-shikimate is a substrate for these two enzymes and thus can be converted into the inhibitor for chorismate synthase *in vivo*. 6-Fluoro-shikimate shows moderate antibacterial and herbicidal activity (*22*). The phytotoxocity may also be partly attributed to inhibition of EPSP synthase by 6-fluoro-shikimate-3-phosphate.

Inhibitors which are analogues of substrates or products generally have only limited potency as their affinity for the enzyme is usually in the same range as that for substrates or products (1 mM to 1 μM). The inhibition may frequently be overcome by the build up of the natural substrate, especially if it is a primary metabolite.

Affinity Labels. The affinity label combines the features of a group specific reagent with a substrate analogue. While possessing structural features that impart an affinity for the enzyme to form a reversible complex, this type of inhibitor also includes a moiety that is chemically reactive toward an amino acid side chain. Because of the reversible complex formed prior to chemical modification, affinity labels can show an enhanced reactivity and specificity toward the target enzyme over group specific reagents. However, affinity labels are still inherently reactive and nonspecific modification of proteins is inevitable. For this approach to be successful, the target enzyme must have an appropriately positioned, modifiable amino acid side chain.

L-(αS,5S)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin, AT-125) is an analogue of glutamine (Figure 3). Acivicin has been found to inactivate a number of glutamine-dependent amidotransferases including anthranilate synthase and glutamate synthase by alkylating an active site cysteine residue (*62*). The chemical mechanism for these enzymes involves the formation of an acyl enzyme intermediate with release of ammonia, which is then transferred to the second substrate. In this way organisms can generate ammonia "in situ" and in controlled amounts. Amino transfer reactions of this type (distinct from transaminations) are involved in numerous biosynthetic pathways including those for purines, pyrimidines, amino acids, and cofactors. In the vast majority of these cases, the amino donor is the glutamine amide. The modest herbicidal activity of acivicin, therefore, probably results from inactivation of a number of enzymes. The reasons for the relatively weak *in vivo* activity are not fully understood, but may be due to reaction of acivicin with nonessential sulfhydryls in the plant, either enzyme-bound or protein-free. Since a large number of enzymes, including mammalian enzymes, use glutamine as an amino donor and appear to have

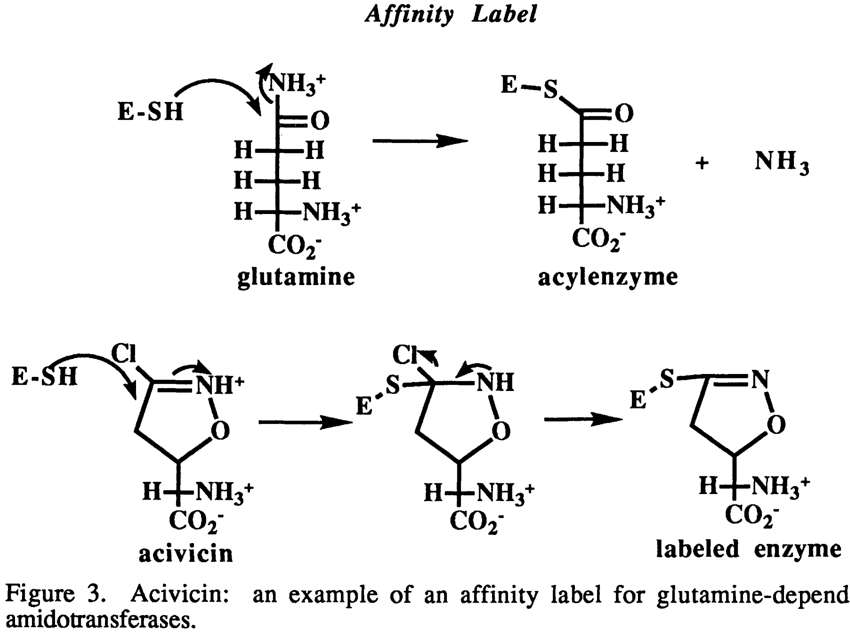


Figure 3. Acivicin: an example of an affinity label for glutamine-dependent amidotransferases.

similar chemical mechanisms which involve a covalent enzyme-thiol-substrate complex, this inhibitor cannot be expected to be very specific. Thus, as in the case of the substrate analogue, it is desirable in this approach for the substrate to be unique to the target enzyme.

Suicide Substrates. Suicide substrates are similar to affinity labels in that inhibition involves initial formation of a reversible complex, due to the similarity of the inhibitor to the substrate, followed by time-dependent formation of an irreversible complex, usually through covalent bond formation with an appropriately positioned amino acid side chain. In contrast to the affinity label, a suicide substrate is not inherently reactive and requires activation by the target enzyme before an irreversible complex is formed. These inhibitors are inherently more selective than affinity labels as they are less reactive in their unactivated form. Since the enzyme participates in its own inactivation, these inhibitors are known by various names such as enzyme-activated, irreversible inhibitors (*63*), suicide substrates (*64*), kcat inhibitors (*65*), and Trojan horse reagents (*66*).

Suicide inhibitors are typically characterized by their turnover to kill ratio, the average number of suicide substrates transformed and released by the enzyme before inactivation occurs. For an ideal suicide substrate, the turnover to kill ratio would be zero, where every transformed inhibitor results in enzyme inactivation with no release of product. Release of the transformed inhibitor, which is now a reactive species, has the potential to modify and thus inactivate other enzymes with loss of specificity.

One example of a suicide substrate is propargyl glycine (Figure 4). Propargyl glycine produces severe growth inhibition of *Lemna paucicostata* at 100 nM which is completely reversed by cystathionine and methionine (*67*). These results along with bacterial studies suggest that the herbistatic and bacteristatic properties of this naturally occurring amino acid are due to inactivation of cystathionine-γ-synthase. Cystathionine-γ-synthase is in the methionine biosynthetic pathway and catalyzes a γ-replacement reaction in which cysteine is coupled with O-phosphohomoserine in plants (*68*) or with O-succinyl homoserine in bacteria to produce cystathionine and inorganic phosphate or succinate, respectively. In the absence of a thiol acceptor, the bacterial enzyme carries out a γ-elimination reaction with O-succinyl-L-homoserine at 1/5 the rate of the normal reaction to yield 2-ketobutyrate, ammonia and succinate (*69*). Exchange of the protons in the α- and β-position of the substrate was observed when the reaction was carried out in D_2O in the presence or absence of thiol (*69*). The mechanism proposed for the inactivation requires the initial formation of an α-carbanion which is common to most pyridoxal 5'-phosphate (PLP) dependent enzymes. However, both the exchange results and the electronic requirements for a γ-elimination reaction suggest the formation of a β-carbanion as well. It is the formation of the β-carbanion adjacent to the acetylene linkage which is thought to trigger inactivation by propargyl glycine through the formation of an electrophilic allene. Once formed, the allene should be a prime target for attack by nucleophilic bases at the PLP active site. The selectivity of propargyl glycine truly lies in the mechanism carried out by the enzyme and not in its structural similarity to normal substrates. A majority of PLP enzymes form α-carbanions, however, they are not inactivated by propargyl glycine because formation of the α-carbanion leaves the acetylenic functionality still unreactive. Only those rare PLP enzymes which can form β-carbanions will be inactivated by propargyl glycine. With a turnover to kill ratio of approximately 4, propargyl glycine is a particularly effective suicide inactivator (*70*).

Reaction Intermediate Analogues. Transition-state or reaction intermediate analogues are stable analogues of labile enzymic reaction intermediates. To the extent that the intermediate is unique, this type of inhibitor can be quite selective. A number of thorough reviews on transition state analogue theory are available (*71, 72*). Since enzymes tend to bind reaction intermediates more tightly than either substrates or products during the course of a chemical transformation, these inhibitors should be much more potent than simple substrate analogues. These molecules are generally not

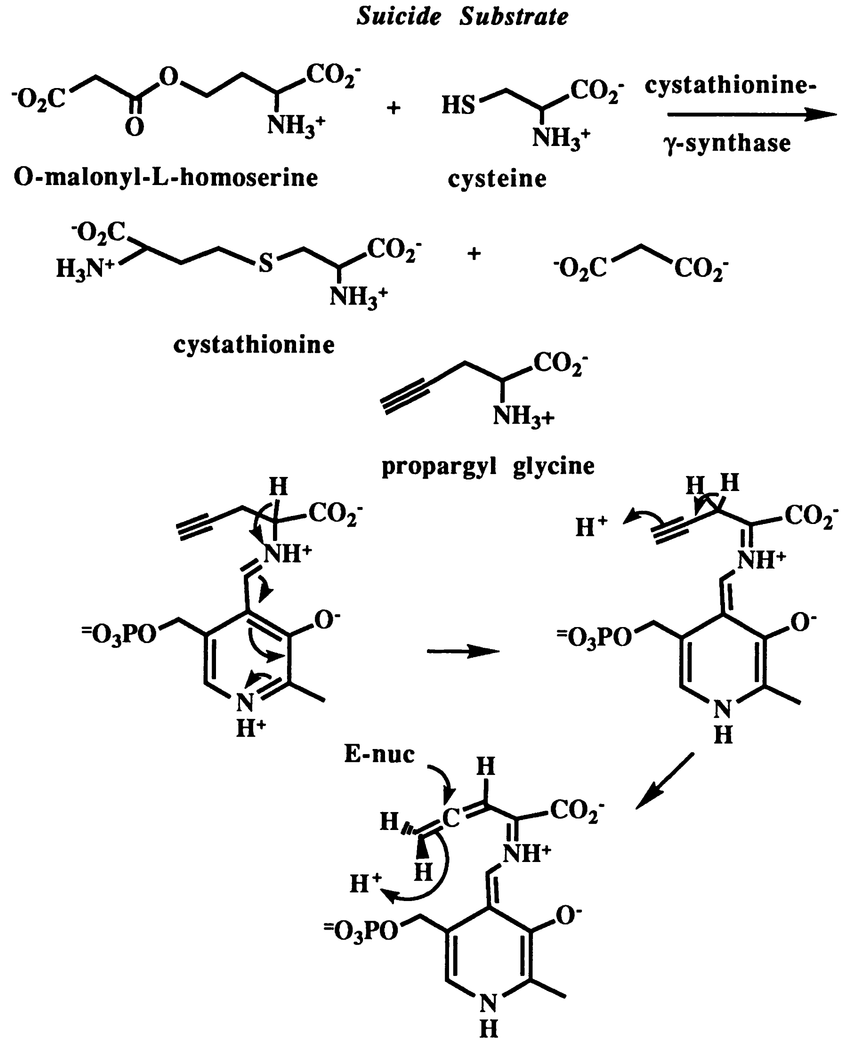


Figure 4. An example of a suicide substrate: propargyl glycine and the postulated mechanism of inactivation of cystathionine-γ-synthase.

chemically reactive and thus there is no indiscriminate labeling of undesired proteins as with affinity labels. The inhibition is not dependent on covalent bond formation so there is no requirement for an appropriately placed nucleophile as with affinity labels and suicide substrates. Since the inhibition is not covalent, the inhibitor can dissociate from the enzyme and thus the inhibition is reversible. The inhibition, then, is a balancing of the association rate and the dissociation rate of a compound. Effective inhibitors of this type often, but not always, display time-dependent inhibition kinetics by virtue of having slow dissociation rates. Half times for dissociation can be as slow as 1-14 years (*73*). While slow dissociation rates are desirable, slow association rates are to be avoided as they increase the amount of time required before the onset of inhibition *in vivo*.

N-isopropyl oxalyl hydroxamate (IpOHA) is a potent inhibitor of ketol acid reductoisomerase (KARI) (*12*). KARI catalyzes the reversible conversion of 2-aceto-2-hydroxybutyrate to 2,3-dihydroxy-3-methylpantanoate or 2-acetolactate to 2,3-dihydroxy-3-methylbutyrate (Figure 5, top). The reaction proceeds in two steps with rearrangement of a β-keto-α-hydroxy-α-alkyl acid to an α-keto-β-hydroxy-β-alkyl acid via a 1,2-methyl shift followed by nucleotide-mediated reduction to a 2,3-diol. The most thoroughly characterized of the oxalyl hydroxamates is the N-isopropyl derivative. The inhibitor has a dissociation constant of 22 pM under optimal conditions in the presence of Mg^{2+} and NADPH. The inhibition is time-dependent and characterized by an association rate of 5.9×10^4 $M^{-1}s^{-1}$ and a dissociation rate of 1.3×10^{-6} s^{-1} with a half time for release of 6.2 days. In the absence of NADPH, the dissociation half time is 2 h. Time-dependent inhibition is not observed in the absence of Mg^{2+}. These results are analogous to the requirements for the rearrangement step which is three times more rapid in the presence of NADPH and absolutely dependent on Mg^{2+}. These results strongly suggest that IpOHA is a potent inhibitior of KARI by virtue of its similarity to the rearrangement transition state. Although the predominant tautomer of IpOHA does not resemble the likely transition state for the rearrangement reaction, the iminol tautomer, which should predominate when the inhibitor chelates Mg^{2+}, has a number of similar structural features. During the rearrangement both hydroxyl-bearing carbons would have partial carbonyl character as well as partial positive charge, and the migrating methyl group would have carbanion character. To the extent that Mg^{2+} is required for the rearrangement and coordinates both oxygens to facilitate the reaction, the chelating properties of the iminol tautomer form of the inhibitor should give enhanced binding.

While IpOHA was designed for this enzyme *de novo*, Hoe 704 from Hoechst was found empirically to be a potent inhibitor of KARI with a K_i of 0.8 μM (*13*). This inhibitor, while not as potent as IpOHA, has characteristics in common with the rearrangement transition state, including partial positive charge on phosphorus and negative charge on oxygen as well as being a potent metal chelator. As discussed earlier, neither compound produced potent herbicidal effects in our hands.

A herbicidally important enzyme which is inhibited by a transition state analogue is glutamine synthetase (GS) (Figure 5, bottom). GS catalyzes the ATP-dependent formation of glutamine from glutamate and ammonia and is the target of the postemergent herbicides glufosinate and bialaphos. The active component of these herbicides is L-phosphinothricin. To understand the inhibition by this compound, one must understand the chemical mechanism of the enzyme. The first step in the reaction involves the transfer of the γ-phosphate of ATP to the γ-carboxylate of glutamate to form the high energy intermediate γ-glutamyl phosphate. This intermediate, which is not released from the enzyme, undergoes ammonolysis in which a phosphorylated tetrahedral intermediate is formed which then eliminates inorganic phosphate to form glutamine. Phosphinothricin is a glutamate analog which contains a tetrahedral phosphinate and therefore resembles the tetrahederal intermediate, except that it lacks the phosphate. Phosphinothricin inactivation of GS from *E. coli* and various plant sources is time dependent and competitive with glutamate. Inactivation has an absolute requirement for ATP. Exposure of the inhibitor-ATP-enzyme complex to denaturation under basic conditions with triethanolamine releases phosphorylated phosphinothricin and ADP which were identified by NMR (Schineller, J., Villafranca, J.J., The Pennsylvania State University, unpublished data). These results indicate that during inactivation the γ-phosphate of ATP is transferred to the phosphinate of phosphinothricin. Thus, the inactive species is the very tight complex formed between enzyme, ADP, and the phosphorylated inhibitor. Rapid-quench kinetic experiments have shown that phosphoryl transfer is fast compared to the association rate for the inhibitor (*74*).

A variation on this theme is the bisubstrate analogue approach. For multi-substrate enzymes, the covalent linkage of two substrates may lead to surprisingly potent

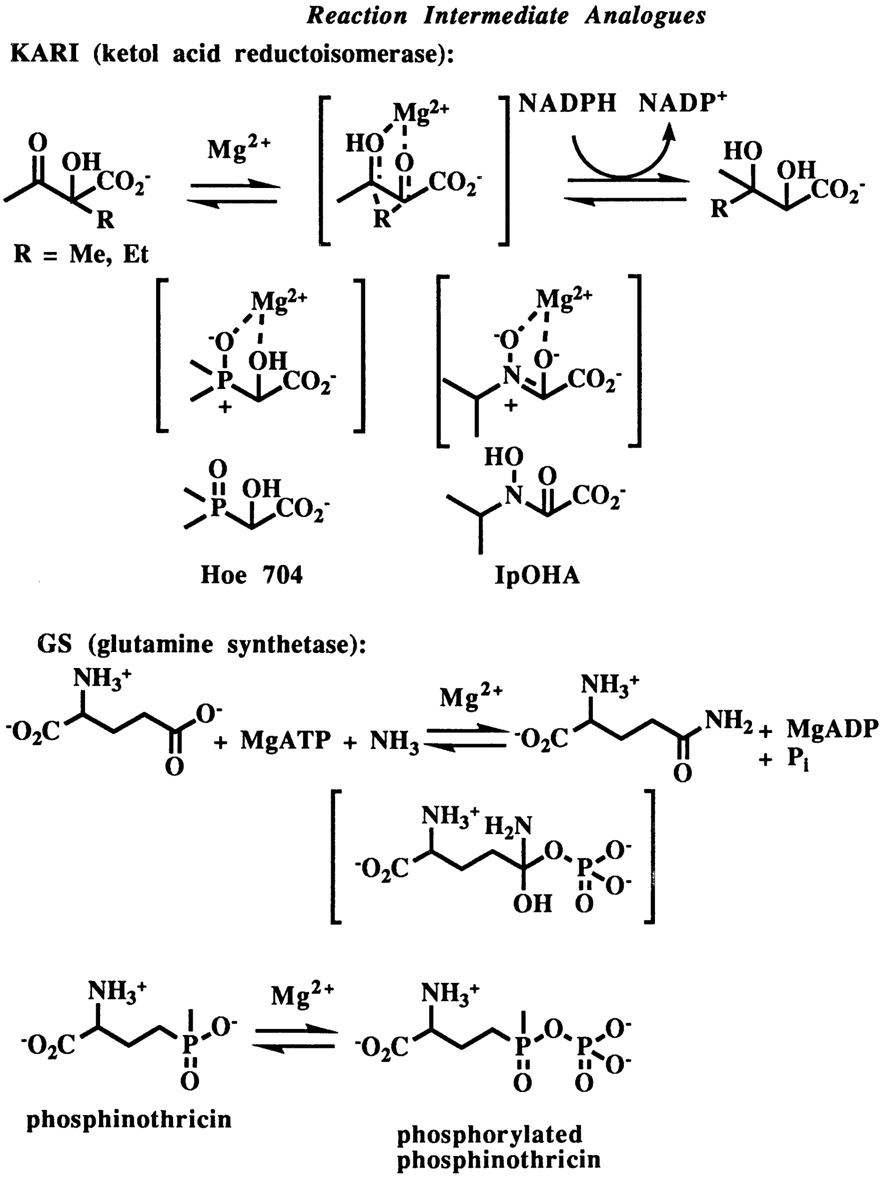

Reaction Intermediate Analogues
KARI (ketol acid reductoisomerase):
R = Me, Et
Mg2+
NADPH NADP+
Hoe 704
IpOHA
GS (glutamine synthetase):
+ MgATP + NH3
+ MgADP + Pi
phosphinothricin
phosphorylated phosphinothricin

Figure 5. Examples of reaction intermediate analogue inhibitors of ketol acid reductoisomerase (top) and glutamine synthetase (bottom).

inhibition. To the extent that the two substrates have a relative orientation resembling that normally found on the enzyme, the affinity of a bisubstrate analogue can exceed that expected based on the enzyme's affinity for the two individual substrates. One basic requirement of this approach is that the two substrates must normally be bound to the enzyme at the same time. In other words, the kinetic mechanism of the enzyme must be sequential rather than ping pong for this approach to work.

N-(phosphonoacetyl)-L-aspartate (PALA) is a potent, bisubstrate analogue inhibitor for aspartate transcarbamylase (*75*) (Figure 6, top). Aspartate transcarbamylase catalyzes the condensation of carbamoylphosphate with L-aspartate to produce N-carbamoylaspartate. The chemical mechanism for this enzyme is thought to involve the initial formation of a tetrahedral carbinolamine intermediate between aspartate and carbamoyl phosphate which then dehydrates to yield the product. PALA is similar to the tetrahedral intermediate that would be formed by attack of the amino group of L-aspartate on the carbonyl group of carbamoyl-phosphate. However, unlike reaction intermediate analogues of tetrahedral adducts, PALA maintains a trigonal carbon at the position corresponding to the tetrahedral carbon of the carbamoylphosphate-L-aspartate adduct. PALA is a potent inhibitor of aspartate transcarbamylase, with an inhibition constant of 10 nM. Several analogues of PALA including two with tetrahedral geometry (sulfonamides) have been described; however, none of the analogues reported thus far are better inhibitors of aspartate transcarbamylase (*76*).

PALA and the analogous inhibitor for ornithine transcarbamoylase, N-(phosphonoacetyl)-L-ornithine (PALO), have been shown to be potent inhibitors for the enzymes from various plant sources with K_i's in the nM range (*77*) (Figure 6, bottom). Ornithine transcarbamoylase is of particular interest as it is known to be the site of action for the chlorosis-inducing tripeptide, phaseolotoxin (*78*). However, both PALO and PALA are only weakly herbicidal (*77*). The reasons for their lack of efficacy are unknown, as no metabolic studies have been reported for these compounds.

Extraneous Site Inhibitors. Extraneous site inhibitors are the most recently recognized class of enzyme inhibitors. These inhibitors can show very tight binding and do not structurally resemble substrates, cofactors, or allosteric effectors. They are termed extraneous site inhibitors because they bind either entirely, or to a large extent, to some site outside of or extraneous to the enzyme active site (*79*).

Extraneous site inhibitors are exemplified by two distinct chemical classes of grass selective herbicides; the aryloxyphenoxypropionic acids typified by diclofop and the cyclohexanediones typified by clethodim (for a recent review see (*56*))(Figure 7, top). In susceptible plants, both classes of herbicides were recently found to be potent, reversible inhibitors of the putative rate limiting enzyme in lipid biosynthesis, acetyl-CoA carboxylase (ACC). The K_i values for diclofop and clethodim with wheat ACC are 15 and 60 nM, respectively. Inhibition and herbicidal activity is stereoselective for the (R)-enantiomer of the aryloxyphenoxypropionates. ACC catalyzes the biotin-dependent carboxylation of acetyl-CoA in two physically and kinetically distinct catalytic sites. In the first partial reaction the enzyme catalyzes the carboxylation of covalently bound biotin in the presence of MgATP and bicarbonate. The carboxybiotin intermediate undergoes nucleophilic attack by the enzyme-generated carbanion of acetyl-CoA at the second catalytic site, releasing malonyl-CoA and recycling the biotin cofactor. Although inhibition of the wheat enzyme by clethodim and diclofop is noncompetitive versus each of the substrates (MgATP, bicarbonate, and acetyl-CoA), both herbicides are nearly competitive versus acetyl-CoA since the level of inhibition is most sensitive to the acetyl-CoA concentration (*80, 81*). Inhibition studies with isotope exchange and partial reactions catalyzed at each separate site showed that both classes of herbicides interfered with only the carboxyltransfer site and did not affect the biotin carboxylation site (*82, 83*). Double inhibition studies suggested that the two herbicide classes are mutaually exclusive and that only the thio ester region of malonyl-CoA and acetyl-CoA overlaps with some structural feature of the herbicides, presumably the small alkyl side chains that are required for good herbicidal properties (*82, 83*). Recent studies with the CoA thio ester conjugates of the aryloxyphenoxypropionates confirms that the CoA pocket is distinct from the herbicide pocket. In this regard the CoA esters of diclofop and fluazifop were from 20 to 425-fold more potent than the corresponding unconjugated herbicides as inhibitors of rat liver (*29*) and wheat ACC (Rendina, A.R.; Taylor, W.S.; Hixon, M.S, E.I. Du Pont de Nemours, unpublished data). Taken together, these observations strongly

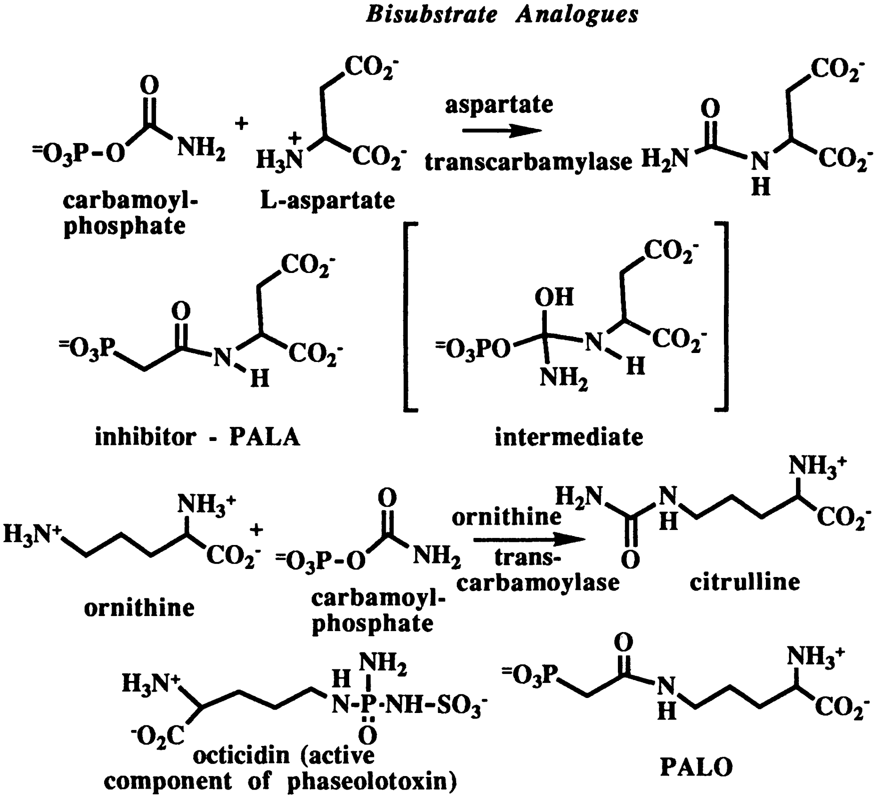

Bisubstrate Analogues
carbamoyl-phosphate
L-aspartate
aspartate transcarbamylase
inhibitor - PALA
intermediate
ornithine
carbamoyl-phosphate
ornithine trans-carbamoylase
citrulline
octicidin (active component of phaseolotoxin)
PALO

Figure 6. Bisubstrate analogue inhibitors for aspartate transcarbamylase (top) and ornithine transcarbamoylase (bottom).

suggest that the aryloxyphenoxypropionate and cyclohexanedione herbicide binding domains only partially overlap with the active site of ACC and that a large portion of the herbicide structures occupies an extraneous site.

The sulfonylurea and imidazolinone herbicides are other examples of extraneous site inhibitors (Figure 7, bottom). These extremely low dosage and nontoxic herbicides inhibit the first common enzyme in branched chain amino acid biosynthesis, acetolactate synthase (ALS) (*11, 84-90*). This enzyme catalyzes the thiamine pyrophosphate (TPP) dependent condensation of two molecules of pyruvate to form acetolactate or the condensation of pyruvate and 2-ketobutyrate to form α-aceto-α-hydroxybutryate. The enzyme requires TPP, flavin adenine dinucleotide (FAD) and Mg^{2+} for catalysis. The FAD plays no redox role in the reaction. Sulfometuron methyl is a slow-binding sulfonylurea inhibitor of ALS with a final K_i of 82 nM for ALS isozyme II from *Salmonella typhimurium*. This slowly reversible complex is formed only in the presence of all cofactors and pyruvate (*91*). Besides requiring pyruvate for tight binding, the inhibitor is competitive with pyruvate. These two seemingly contradictory effects of pyruvate are probably due to the two different molecules of pyruvate involved in the reaction. The first pyruvate adds to the thiamine pyrophosphate to give the intermediate form of the enzyme to which sulfometuron methyl binds. The herbicide is nearly competitive with the second molecule of pyruvate and must therefore overlap at least partially with the second pyruvate binding site.

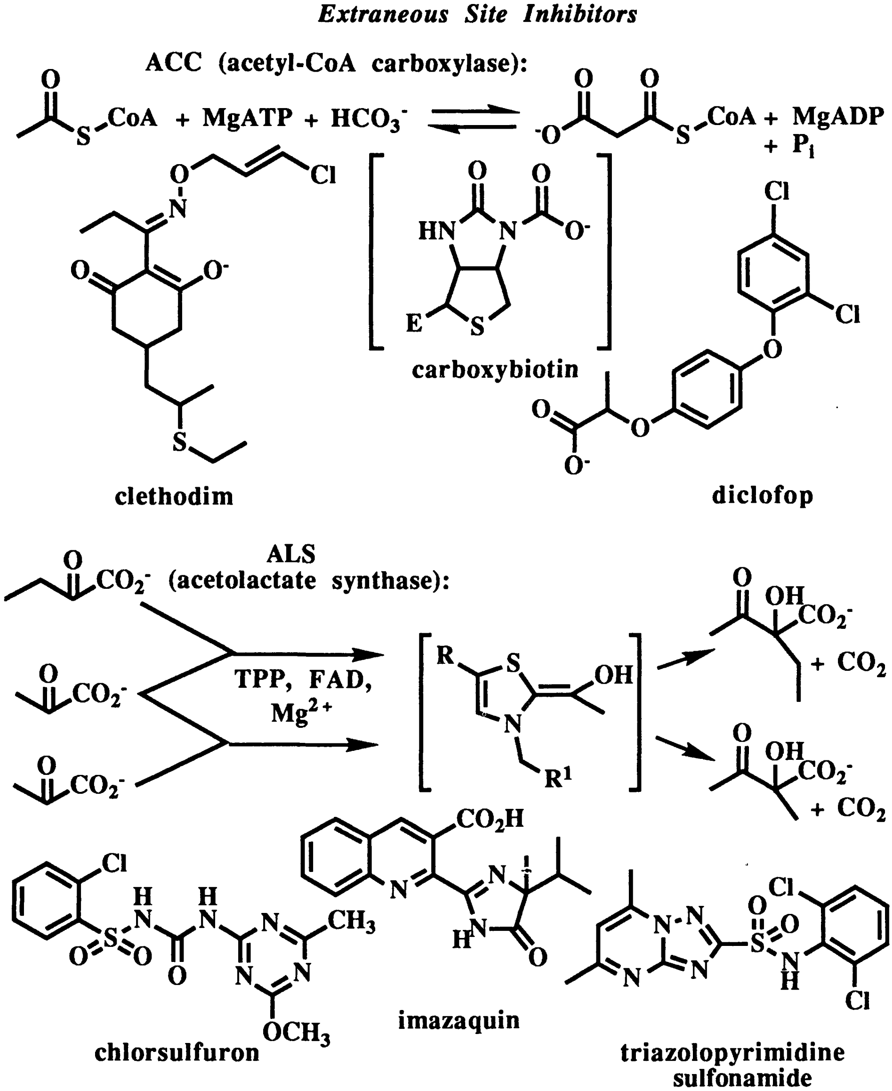

Extraneous Site Inhibitors
ACC (acetyl-CoA carboxylase):
CoA + MgATP + HCO3⁻
CoA + MgADP + Pi
carboxybiotin
clethodim
diclofop
ALS (acetolactate synthase):
TPP, FAD, Mg2+
+ CO2
+ CO2
chlorsulfuron
imazaquin
triazolopyrimidine sulfonamide

Figure 7. Examples of extraneous site inhibitors: acetyl-CoA carboxylase (top) and acetolactate synthase (bottom).

On the other hand the imidazolinone, imazaquin, is nearly an uncompetitive inhibitor of ALS with respect to pyruvate, indicating that its binding site does not overlap with the second pyruvate binding site (*89*). The key to identifying the nature of the binding site for these herbicides came with a comparison of the sequence of *poxB* gene of pyruvate oxidase (*92*) and that for the large subunit of the three isozymes of ALS *ilv*B (*93, 94*), *ilvG* (*95*), and *ilvI* (*96*). Unlike ALS, the FAD in pyruvate oxidase serves a redox role. After oxidation of pyruvate, the reduced FAD is reoxidized by ubiquinone-40. The interaction of ubiquinone-40 with pyruvate oxidase is tightest in the presence of pyruvate. These conditions are analogous to those required for effective herbicide interaction with ALS. The substantial sequence homology between *poxB* and the ALS isozymes demonstrates that the two enzymes are evolutionarily related and suggest the possibility that a vestigial ubiquinone binding site remains in ALS (*79*). Binding of the sulfonylurea herbicides to this leftover, unused quinone binding site of ALS is responsible for their inhibition. Consistent with this hypothesis, ALS is inhibited by the water soluble quinones, Q_O and Q_1. The inhibition is time dependent and Q_O can displace bound radiolabeled sulfonylureas from the enzyme (ALS isozymes I and III only) (*79*).

The major drawback of extraneous site inhibitors is that resistance can develop readily. Since the site where the inhibitor binds is usually not essential for the function of the enzyme, selection pressures can produce mutations in that region which can eliminate the inhibition without having deleterious effects on catalytic efficiency. The nature of these inhibitors makes them nearly impossible to design from knowledge of the enzyme's mechanism. They are most readily discovered through screening although knowledge of the target can greatly facilitate optimization of activity. The application of molecular modeling and knowledge of the crystal structure of the enzyme-inhibitor complex can also facilitate optimization of activity (for a recent success in drug design see (*97*)).

Of the types of inhibitors described, the reaction intermediate analogues have the greatest promise for use in herbicide design. These inhibitors display both great potency and selectivity for the target enzyme. The general strategy for design of a reaction intermediate analogue can frequently be adapted to other enzymes with similar mechanisms. However, because of the specificity of reaction intermediate analogues, there may be only a narrow range of structures that yield potent inhibition (e.g., glyphosate and phosphinothricin). Consequently, this design approach is likely to lead to nonselective, broad spectrum herbicides. The high degree of structural stringency makes it less likely that crop safety will occur via differential detoxification. To achieve crop tolerance, the strategy will be to utilize genetic engineering or plant breeding approaches early in the process to introduce herbicide resistance. Unlike the extraneous site inhibitors, natural resistance to this type of inhibition is rare. Since these inhibitors bind to the enzyme active site and are designed with knowledge of the function of the enzyme, mutations at the active site which might effectively overcome the inhibition are very likely to affect the catalytic efficiency of the enzyme in an unacceptable manner. These inhibitors are not difficult to conceive, but require detailed mechanistic knowledge of the enzyme for their successful design.

Translation of *In Vitro* Activity to *In Vivo* Efficacy

Presumably, having followed the tenets outlined in this article, a suitable, validated enzyme target has been successfully chosen and a potent inhibitor has been designed and synthesized for that enzyme. Ideally, the inhibitor should produce potent phytotoxic effects; however, in many instances additional barriers must be overcome, namely, uptake, transport (both inter- and intra-cellular), and metabolic detoxification of the compound. All of these hurdles can be addressed using proherbicidal approaches (*98*). Which strategy to employ will obviously depend on which factor is limiting the delivery of intact inhibitor to the target site. Therefore, it is very important to prepare radiolabeled inhibitor at an early stage to facilitate uptake, translocation, and metabolism studies. These follow-up studies are extremely important considering the amount of effort invested in validating the target and obtaining the mechanistic knowledge to design inhibitors.

Rational approaches to uptake, translocation, and selective metabolism have been reviewed recently and these works and the references therein should be consulted for a more detailed discussion (*99-101*). The proper balance of physical properties plays a major role in uptake and transport, so some consideration of the known factors must be applied early in the design process, i.e., in selecting targets to inhibit.

Proherbicides could be made to balance lipophilicity or reduce charge, but these approaches are limited because the "pro" part of the molecule generally must be labile enough either chemically or biochemically to release the active inhibitor for proper fit in the target site. A balance between enhanced uptake and translocation and the required speed of release of the active component must be achieved. Some examples of proherbicidal approaches to masking active functional groups are to use esters of carboxylates or other masking groups (e.g., aryloxyphenoxypropionates (*102*)); use extended acids to take advantage of β-oxidatative cleavage back to the desired shorter carboxylate functionality (e.g., 2,4-dichlorophenoxybutyrate(*103*)); and to use ketones for amino groups, presumably the plant transaminases can add the amino group (e.g., α-ketophosphinothricin (*104*)). Additional proherbicide approaches are reviewed in (*98*). For enhanced cellular uptake it may be possible to attach the inhibitor to structural moeities that facilitate uptake: e.g., water soluble vitamins such as folate, vitamin B_{12},and biotin (*105-107*), sugars (*108*), or nicotinic acids (*109, 110*). Again, either the target site must accomodate these additional structures or the proper balance must be established between the benefits of enhanced uptake and the rapidity of conversion of the proherbicide to the active component.

With luck, selectivity between the crop plant and the weeds can be achieved with a target-site designed inhibitor requiring no further structural modifications. But rather than to rely upon luck, it is far preferable to elucidate the typical detoxification mechanisms in various plants and use this information to advantage early in the design process. If detoxification cannot be avoided in the design of a specific inhibitor, then it becomes important to elucidate the detoxification pathway, and concurrently design selective inhibitors of detoxification. In this approach, inhibitors of the detoxifying pathway are used in the hope that differences in sensitivities of detoxifying enzymes will exist between crops and weeds. Such a strategy has been employed with known herbicides. For example, tridiphane, a synergist for triazine herbicides, blocks glutathione S-transferase in susceptible weeds but not in corn. Tridiphane itself is conjugated to glutathione to form a more active inhibitor of glutathione S-transferase. This enzyme would otherwise detoxify triazine herbicides in both crops and weeds (*111*).

One of the most difficult processes is to predict how functional groups are metabolized and which ones get transported well, especially when these groups are part of a novel structure. For true rational design of crop selective herbicides to be realized, more basic research is required on the substrate specificities of plant xenobiotic metabolizing enzymes and the factors important for the induction of those activities.

Conclusions

Target-site directed research will ultimately succeed in producing novel pesticides. The approach has the potential to produce extremely low use rate compounds that are environmentally friendly and nontoxic. Target-site design differs from traditional screening approaches in that it is potentially less labor intensive and more knowledge intensive. The main advantage is that it should dramatically reduce the number of compounds and the time required to discover new herbicides. The approach also tends to investigate areas of chemistry that have previously been under-explored or exploited, namely water-soluble chemicals. As with all newborns, the target-site directed approach will need to be encouraged in order to mature into a productive adult.

Acknowledgments

We thank Vern Wittenbach, Wendy Taylor and Jim Kerschen for many helpful discussions in the preparation of this manuscript.

Literature Cited

1. Duke, S.A.; Abbas, H.K.;Boyette, C.D. *Proc. Brighton Crop Protection Conf.-Weeds* **1991**, *2*, 155-164.
2. Ciskanik, L.M.; Schloss, J.V. *Fed. Proc.* **1986**, *45*, 1607.
3. Alberg, D.G.; Bartlett, P.A. *J. Am. Chem. Soc.* **1989**, *111*, 2337-2338.
4. Johnson, C.R.; Boettcher, B.R.; Cherpeck, R.E.; Dolson, M.G. *Bioorg. Chem.* **1990**, *18*, 154-159.
5. Logusch, E.W.; Walker, D.M.; McDonald, J.F.; Franz, J.E. *Biochemistry* **1990**, *29*, 366-372.
6. Logusch, E.W.; Walker, D.M.; McDonald, J.F.; Franz, J.E. *Plant Physiol.* **1991**, *95*, 1057-1062.
7. Logusch, E.W.; Walker, D.M.; McDonald, J.F.; Franz, J.E. *Biochemistry* **1989**, *28*, 3043-3051.
8. Logusch, E.W.; Walker, D.M.; McDonald, J.F.; Leo, G.C.; Franz, J.E. *J. Org. Chem.* **1988**, *53*, 4069-4074.
9. LaRossa, R.A.; Falco, S.C. *Trends Biotechnol.* **1984**, *2*, 158-161.
10. LaRossa, R.A.; Van Dyk, T.K. *BioEssays* **1987**, *7*, 125-129.
11. LaRossa, R.A.; Schloss, J.V. *J. Biol. Chem.* **1984**, *259*, 8753-8757.
12. Aulabaugh, A.; Schloss, J.V. *Biochemistry* **1990**, *29*, 2824-2830.
13. Schulz, A.; Sponemann, P.; Kocher, H.; Wengenmayer, F. *FEBS Lett.* **1988**, *238*, 375-378.
14. Westkaemper, R.B.; Zenk, P.C. *Eur. J. Med. Chem.* **1988**, *23*, 233-236.
15. Pirrung, M.C.; Ha, H.-J.; Holmes, C.P. *J. Org. Chem.* **1989**, *54*, 1543-1548.
16 Pirrung, M.C.; Holmes, C.P.; Horowitz, D.M.; Nunn, D.S. *J. Am. Chem. Soc.* **1991**, *113*, 1020-1025.
17. Steinrucken, H.; Amrhein, N. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1207-1212.
18. Montchamp, J.-L.; Frost, J.W. *J. Am. Chem. Soc.* **1991**, *113*, 6296-6298.
19. Myrvold, S.; Reimer, L.M.; Pompliano, D.L.; Frost, J.W. *J. Am. Chem. Soc.* **1989**, *111*, 1861-1866.
20. Piehler, L.T.; Montchamp, J.-L.; Frost, J.W.; Manly, C.J. *Tetrahedron* **1991**, *47*, 2423-2432.
21. Bugg, T.D.H.; Abell, C.; Coggins, J.R. *Tet. Lett.* **1988**, *29*, 6783-6786.
22. Sutherland, J.K.; Watkins, W.J.; Snow, G.A.; Davies, G.M. *Patent: EP 393923* **1990**.
23. Balasubramanian, S.; Davies, G.M.; Coggins, J.R.; Abell, C. *J. Am. Chem. Soc.* **1991**, *113*, 8945-8946.
24. Bartlett, P.A.; Nakagawa, Y.; Johnson, C.R.; Reich, S.H.; Luis, A. *J. Org. Chem.* **1988**, *53*, 3195-3210.
25. Clarke, T.; Stewart, J.D.; Ganem, B. *Tetrahedron* **1990**, *46*, 731-748.
26. Wood, H.B.; Buser, H.-P.; Ganem, B. *J. Org. Chem.* **1992**, *57*, 178-184.
27. Wittenbach, V.A.; Aulabaugh, A.;Schloss, J.V. In *Examples of Extraneous Site Inhibitors and Reaction Intermediate Analogs: Acetolactate Synthase and Ketol-Acid Reductoisomerase*; Frehse, H., Ed.; Pesticide Chemistry; VCH: Weinheim, 1991; pp 151-160.
28. Bouzayen, M.; Hamilton, A.; Picton, S.; Barton, S.; Grierson, D. *Biochem. Soc. Trans.* **1992**, *20*, 76-79.
29. Kemal, C.; Casida, J.E. *Life Sciences* **1992**, *50*, 533-540.
30. Schneider, T.; Dinkins, R.; Robinson, K.; Shellhammer, J.; Meinke, D.W. *Develop. Biol.* **1989**, *131*, 161-167.
31. Wallsgrove, R.M.; Risiott, R.; King, J.; Bright, S.W.J. *Plant Sci.* **1986**, *43*, 109-114.
32. Horsch, R.B.; King, J. *Planta* **1983**, *159*, 12-17.

33. Lea, P.J.; Blackwell, R.D.; Murray, A.J.S.; Joy, K.W. *Recent Adv. Phytochem.* **1989**, *23*, 157-189.
34. Blackwell, R.D.;Murray, A.J.S.;Lea, P.J.*J. Exp. Botany* **1987**,*38*,1799-1809.
35. Rupp, W.; Finke, M.; Bieringer, H.;Langeleuddeke, P. *Patent: Ger. Offen. DE 2717440* **1977**.
36. Bayer, E.; Gugel, K.H.; Hagele, H.; Hagenmaier, H.; Jessipow, S.; Konig, W.A.; Zahner, H. *Helv. Chim. Acta* **1972**, *55*, 224-239.
37. Rothstein, S.J.; DiMaio, J.; Strand, M.; Rice, D. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 8439-8443.
38. Sandler, S.J.; Stayton, M.; Townsend, J.A.; Ralston, M.L.; Bedrock, J.R.; Dunsmuir, P. *Plant Mol. Biol.* **1988**, *11*, 301-310.
39. Delauney, A.J.; Tabaeizadeh, Z.; Verma, D.P.S. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 4300-4304.
40. Ecker, J.R.; Davis, R.W. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 5372-5376.
41. van der Krol, A.R.; Mur, L.A.; de Lange, P.; Gerats, A.G.M.; Mol, J.N.M.; Stuitje, A.R. *Mol. Gen. Genet.* **1990**, *220*, 204-212.
42. van der Krol, A.R.; Stuitje, A.R.; Mol, J.N.M. In *Antisense Nucleic Acids and Proteins*, Mol, J.N.M.; van der Krol, A.R., Eds.; Marcel Dekker: New York, 1991; pp 125-140.
43. Schuch, W., Bird, C.R.; Ray, J.; Smith, C.J.S.; Watson, C.F.; Morris, P.C.; Gray, J.E.; Arnold, C.; Seymour, G.B.; Tucker, G.A.; Grierson, D. *Plant Mol. Biol.* **1989**, *13*, 303-311.
44. Smith, C.J.S.; Watson, C.F.; Ray, J.; Bird, C.R.; Morris, P.C.; Schuch, W.; Grierson, D. *Nature* **1988**, *334*, 724-726.
45. Cornelissen, M.; Vandewiele, M. *Nuc. Acids Res.* **1989**, *17*, 833-843.
46. Robert, L.S.; Donaldson, P.A.; Ladaique, C.; Altosaar, I.; Arnison, P.G.; Fabijanski, S.F. *Plant Mol. Biol.* **1989**, *13*, 399-409.
47. Cannon, M.; Platz, J.; O'Leary, M.; Sookdeo, C.;Cannon, F. *Plant Mol. Biol.* **1990**, *15*, 39-47.
48. Visser, R.G.F.; Somhorst, I.; Kuipers, G.J.; Ruys, N.J.; Fenstra, W.J.; Jacobsen, E. *Mol. Gen. Genet.* **1991**, *225*, 289-296.
49. Hudson, G.S.; Evans, J.R.; von Caemmerer, S.; Arvidsson, Y.B.C.; Andrews, T.J. *Plant Physiol.* **1992**, *98*, 294-302.
50. Rodermel, S.R.; Abbott, M.S.; Bogorad, L. *Cell* **1988**, *55*, 673-681.
51. Oeller, P.W.; Min-Wong, L.; Taylor, L.P.; Pike, D.A.; Theologis, A. *Science* **1991**, *254*, 437-439.
52. Hamilton, A.J.; Lycett, G.W.; Grierson, D. *Nature* **1990**, *346*, 284-287.
53. Elkind, Y.; Edwards, R.; Mavandad, M.; Hedrick, S.A.; Ribak, O.; Dixon, R.A.;Lamb, C.J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9057-9061.
54. Amrhein, N.; Godeke, K.-H. *Plant Sci. Lett.* **1977**, *8*, 313-317.
55. Amrhein, N.; Hollander, H. *Planta* **1979**, *144*, 385-389.
56. Gronwald, J.W. *Weed Sci.* **1991**, *39*, 435-449.
57. Hamm, P.C. In *Herbicides, Fungicides, Formulation Chemistry, Proc. 2nd Int. Congr. Pestic. Chem. (IUPAC)*; Tahori, A. S., Ed.; Gordon and Breach: New York, 1972, Vol. 5; pp 41-64.
58. Jaworski, E.G. *J. Agric. Food Chem.* **1969**, *17*, 165-170.
59. Roland, S.; Ferone, R.; Harvey, R.J.; Styles, V.L.; Morrison, R.W. *J. Biol. Chem.* **1979**, *20*, 10337-10345.
60. Veerasekaran, P.;Kirkwood, R.C.;Parnell, E.W.*Pestic. Sci.***1981**,*12*, 330-338.
61. Balasubramanian, S.; Abell, C.; Coggins, J.R. *J. Am. Chem. Soc.* **1990**, *112*, 8581-8583.
62. Tso, J.Y.; Bower, S.G.; Zalkin, H. *J. Biol. Chem.* **1980**, *255*, 6734-6738.
63. Silverman, R.B. *Mechanism-Based Enzyme Inactivation : Chemistry and Enzymology*; CRC Press: Boca Raton, Florida, 1988, Vol. I & II.
64. Walsh, C.T. *Horiz. Biochem. Biophys.* **1977**, *3*, 36-81.

65. Rando, R.R. *Science* **1974**, *185*, 320-324.
66. Walsh, C. *Tetrahedron* **1982**, *38*, 871-909.
67. Datko, A.H.; Mudd, S.H. *Plant Physiol.* **1982**, *69*, 1070-1076.
68. Datko, A.H.;Giovanelli, J.;Mudd, S.H. *J. Biol. Chem.* **1974**,*249*, 1139-1155.
69. Guggenheim, S.; Flavin, M. *J. Biol. Chem.* **1969**, *244*, 6217-6227.
70. Johnston, M.; Jankowski, D.; Marcotte, P.; Tanaka, H.; Esaki, N.; Soda, K.; Walsh, C. *Biochemistry* **1979**, *18*, 4690-4701.
71. Schloss, J.V. In *Target Sites of Herbicide Action*; Boger, P.H.; Sandmann, G. Eds.; CRC Press: Boca Raton, Florida, 1989; pp 165-245.
72. Schloss, J.V. *Accounts Chem. Res.* **1988**, *21*, 348-353.
73. Kaplan, A.P.; Bartlett, P.A. *Biochemistry* **1991**, *30*, 8165-8170.
74. Abell, L.M.; Villafranca, J.J. *Biochemistry* **1991**, *30*, 6135-6141.
75. Collins, K.D.; Stark, G.R. *J. Biol. Chem.* **1971**, *246*, 6599-6605.
76. Farrington, G.K.; Kumar, A.; Wedler, F.C. *J. Med. Chem.* **1985**, *28*, 1668-1673.
77. Acaster, M.A.; Scott-White, S.; Weitzman, P.D. *J. Exp. Bot.* **1989**,*40*, 1121-1125.
78. Patil, S.S.; Kwok, O.C.H.; Moore, R.E. *Toxicon* **1983**, *Suppl. 3*, 341-344.
79. Schloss, J.V. In *Prospects for Amino Acid Biosynthesis Inhibitors Crop Protection and Pharmaceutical Chemistry* ; Copping, L.G.; Dalziel, J.; Dodge, A.D. Eds.; The British Crop Protection Council: Farnham, 1989, BCPC Monograph No. 42; pp 147-152.
80. Rendina, A.R.; Felts, J.M. *Plant Physiol.* **1988**, *86*, 983-986.
81. Rendina, A.R.; Felts, J.M.; Beaudoin, J.D.; Craig-Kennard, A.C.; Look, L.L.; Paraskos, S.L.; Hagenah, J.A. *Arch. Biochem. Biophys.* **1988**, *265*, 219-225.
82. Rendina, A.R.; Beaudoin, J.D.; Craig-Kennard, A.C.; Breen, M.K. *Proc. Brighton Crop Protection Conf.-Weeds* **1989**, *1*, 163-172.
83. Rendina, A.R.; Craig-Kennard, A.C.; Beaudoin, J.D.; Breen, M.K. *J. Agric. Food Chem.* **1990**, *38*, 1282-1287.
84. Falco, S.C.; Dumas, K.S.; McDevitt, R.E. In *Molecular Form and Function of the Plant Genome*; NATO Advance Studies Institute Ser.: Renesse, The Netherlands,1985, Ser. A; pp 467-478.
85. Falco, S.C.; Dumas, K.S. *Genetics* **1984**, *107*, S30.
86. Falco, S.C.; Dumas, K.S. *Genetics* **1985**, *109*, 21-35.
87. Ray, T.B. *Plant Physiol.* **1984**, *75*, 827-831.
88. Chaleff, R.S.; Mauvais, C.J. *Science* **1984**, *224*, 1443-1445.
89. Shaner, D.L.;Anderson, P.C.;Stidham, M.A.*Plant Physiol.* **1984**,*76*, 545-546.
90. Shaner, D.L.; Anderson, P.C.; Stidham, M.A. *Plant Physiol.* **1984**, *75S*, 50.
91. Schloss, J.V. In *Flavins and Flavoproteins*; Bray, R.C.; Engel, P.C.; Mayhew, S.G. Eds.; Walter de Gruyter: Berlin, 1984; pp 737-740.
92. Grabau, C.; Cronan, J.E., Jr. *Nuc. Acids Res.* **1986**, *14*, 5449-5460.
93. Friden, P.; Donegan, J.; Mullen, J.; Tsui, P.; Freundlich, M. *Nuc. Acids Res.* **1985**, *13*, 3979-3993.
94. Wek, R.C.; Hauser, C.A.; Hatfield, G.W. *Nuc. Acids Res.* **1985**, *13*, 3995-4010.
95. Lawther, R.P.; Calhoun, D.H.; Adams, C.W.; Hauser, C.A.; Gray, J.; Hatfield, G.W. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 922-925.
96. Squires, C.H.; De Felice, M.; Devereux, J.;Calvo, J.M. *Nuc. Acids Res.* **1983**, *11*, 5299-5313.
97. Ealick, S.E.; Babu, Y.S.; Bugg, C.E.; Erion, M.D.;Guida, W.C.;Montgomery, J.A.; Secrist III, J.A. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 11540-11544.
98. Kerr, M.W. In *Herbicide and Plant Metabolism*; Dodge, A.D. Ed.; Cambridge Univ. Press: 1989, Soc. Exp. Biol. Sem. Series Vol. 38; pp 199-210.
99. Coupland, D. *Proc. Brighton Crop Protection Conf.-Weeds* **1991**, *2*, 837-846.

100. Brown, H.M.; Dietrich, R.F.; Kenyon, W.H.; Lichtner, F.T. *Proc. Brighton Crop Protection Conf.-Weeds* **1991**, *2*, 847-856.
101. Owen, W.J. In *Herbicide and Plant Metabolism*; Dodge, A.D. Ed.; Cambridge Univ. Press: 1989, Soc. Exp. Biol. Sem. Series Vol. 38; pp 171-198.
102. Shimabukuro, R.H.; Walsh, W.C.; Hoerauf, R.A. *J. Agric. Food Chem.* **1979**, *27*, 615-623.
103. Wain, R.L. *J. Agric. Food Chem.* **1955**, *3*, 128-130.
104. Pillmoor, J.B.; Wright, K.; Lindell, S.D. *Proc. Brighton Crop Protection Conf.-Weeds* **1991**, *2*, 857-866.
105. Horn, M.A.; Heinstein, P.F.; Low, P.S. *Plant Physiol.* **1990**, *93*, 1492-1496.
106. Low, P.S.; Horn, M.A.; Heinstein, P.F. *Patent: PCT Int. Appl. WO 90 12,095* **1989.**
107. Low, P.S.; Horn, M.A.; Heinstein, P.F. *Patent: PCT Int. Appl. WO 90 12,096, US Appl. 331,816* **1989.**
108. Hitz, W.D.; Card, P.J.; Ripp, K.G. *J. Biol. Chem.* **1986**, *261*, 11986-11991.
109. Hawkes, T.R. In *Prospects for Amino Acid Biosynthesis Inhibitors Crop Protection and Pharmaceutical Chemistry* ; Copping, L.G.; Dalziel, J.; Dodge, A.D. Eds.; The British Crop Protection Council: Farnham, 1989, BCPC Monograph No. 42; pp 131-138.
110. Hawkes, T.R.; Thomas, S.E. In *Biosynthesis of Branched Chain Amino Acids*; Baraak, Z.; Chipman, D.M.; Schloss, J.V., Eds.; VCH: Weinheim, 1991; pp 373-389.
111. Lamoureux, G.L.; Rusness, D.G. *Pestic. Biochem. Physiol.* **1986**, *26*, 323-342.

RECEIVED July 22, 1992

Chapter 3

Engineering Crop Resistance to the Naturally Occurring Glutamine Synthetase Inhibitor Phosphinothricin

H. Müllner, P. Eckes, and G. Donn

Hoechst AG, 6000 Frankfurt am Main 80, Germany

Chemical plant protection will be always needed, but the application of gene technology can reduce the impact of agriculture to the environment and offer new attractive systems for weed control to the farmer. The non-selective herbicide glufosinate exhibit desirable properties, which makes it suitable for weed control in crops. By transferring a microbial resistance gene from the producer of the active principle of glufosinate, sensitive crops like corn, oilseed-rape, soy bean and sugarbeet could be made resistant. In comparison to present, on soil herbicides based weed control systems, the flexibility in the application of the post-emergent foliar herbicide glufosinate in resistant crops comes closer to an ideal system. The introduction of this new system will be another important step towards an agriculture with reduced impact on the environment.

Worldwide there is still a need to increase agricultural efficiency, because in some of the developing countries, the population is rising much faster than productivity. In contrast, in the industrialized countries agriculture is already highly efficient and can produce more than is necessary to feed the population. This is, besides the use of fertilizers and other improvements, to a large part due to the use and the application of efficient agrochemicals, which protect the crop from competition by weeds, attack by insects, and from fungal diseases (Table I).

Table I. Annual Application of Plant Protection Chemicals

Pesticide Class	Worldwide	U.S.
	(kg a.i. x 1000)	
Herbicides	538,000	231,000
Insecticides	350,000	52,000
Fungicides	580,000	66,000

0097–6156/93/0524–0038$06.00/0

However, in the northern hemisphere the amount of applied chemicals and the consequences thereof have led to a new awareness about the environmental consequences of agriculture. There is now an increasing demand for a change in policy to reduce the undesired side effects of intensive agriculture.

In order to maintain an efficient production of food and to reduce the impact on the environment, industry is looking for new chemicals with better ecotoxicological profiles, better activity, and higher selectivity. The sulfonylureas are an example of such a new chemistry which might lead to a substantial reduction of the total amount of applied herbicides. On the other hand industry and scientific institutes are interested in the evaluation and the development of alternative measures which might, in combination with other means of good farming practices, allow an additional reduction of the impact on the environment. Such a system, containing crop rotation, biological and chemical plant protection, as well as the use of improved seeds, is called integrated crop management. This concept is not yet fully developed or generally accepted in agricultural crops.

As part of this integrated system, plant breeding will increase in importance due to the progress in modern techniques of cell biology and molecular biology (1,2).

By these means, it is now possible to transfer genes from various organisms into plants and thereby generate plants resistant to insects (3), fungal pathogens (4) and viruses (5), plants with modified storage products, or plants which are resistant to herbicides with desirable properties, but no selectivity in the crops (6,7).

Glufosinate Tolerant Crops

In the following section, the concept of glufosinate as a selective herbicide will be discussed. We will attempt to answer the question: "Will the use of glufosinate in glufosinate-resistant crops lead to a reduction in the environmental impact of chemical weed control?"

To answer this question adequately, it would be useful to first compare standard weed control practices with a weed control system in glufosinate-resistant crops. This chapter will be divided into three parts, which will cover:

I. Present weed control systems in corn, rape seed, and sugar beet and their limitations

II. Glufosinate, a non-selective herbicide suitable for development of resistant crops

III. Improved post-emergent weed control systems with reduced impact on the environment

Present Weed Control Systems in Corn, Rape Seed, and Sugar Beet and Their Limitations

As examples of present weed control systems, we will focus on corn, oilseed-rape, and sugar beet. These key crops are planted on a substantial part of the area used for farming (Table II).

Table II. Area Planted in Major Crops in 1990

Crop	North America	Europe
	(million ha)	
corn	28.0	13.6
oilseed rape	2.6	3.1
sugar beet	0.5	3.5

The most commonly-used weed control chemicals in corn are atrazine, alachlor, and other triazines and acetanilides (Table III).

Table III. Herbicides Used in Corn

	Application type		Application Rate
Area treated	Preemergence	Postemergence	(kg a.i./ha)
5 - 10 %	-	-	-
20 - 50 %	atrazine, alachlor and/or mixtures with triazines	-	1.5 - 2.5
40 - 75 %	atrazine, alachlor, and/or mixtures	atrazine, cyanazine	3.0 - 5.0

Whereas only less than 10 % of the maize growing area is not treated with herbicides, more than 80 % of the area is treated with acetanilides and triazines in one or two applications. The total amount of active ingredient applied per hectare is in the range of 1.5 to 5 kg. The extensive use of this weed control system has some limitations. The use of residual soil herbicides is still very popular, because they can be applied at the date of sowing and under appropiate soil humidity conditions the weed control is sufficient for the whole growing season.

But under dry weather conditions as well as in soils with a high content of organic matter, the efficacy of the soil herbicides may be not sufficient. Additional applications of herbicides after the emergence of the crop may be necessary. Efficient soil herbicides must be at least partially mobile in the soil and should not be degraded too fast. Otherwise they would not have the desired long lasting herbicidal effect. As a consequence there exists a change that they may migrate in the soil faster than they are degraded, especially in light soils under heavy rainfall conditions. This may lead to groundwater contamination. Because the soil is not protected by a substantial canopy of vegetation during the early stages of crop development, the bare soil is subject to erosion, either by heavy rainfalls or wind.

Table IV. Weed Control Systems in Canola and Oilseed Rape

	Application type		Application Rate (kg a.i./ha)
	Preemergence	Postemergence	
Canola in North-America	trifluralin incorporated into the soil	sethoxidim, diclofop, fluazifop and broad leaf herbicides	1.0 - 3.0
oilseed rape (winter-type) in Europe	trifluralin and metazachlor, carbetamide, dimefuron	metazachlor and carbetamide, dimefuron, napropamide, fluazifop	1.5 - 3.0

Also in oilseed rape, weed control is dominated by the use of residual soil herbicides (Table IV). More than 70 % of the herbicides used in canola in Canada belong to the dinitroanilides-group. In European winter-type rape seed, dinitroaniline herbicides are supplemented by other post-emergent herbicides. The problems of this weed control system are similar to those in corn.

In Canada the loss of soil moisture is regarded as an important problem, as is in all areas the lack of complete control of wild mustard. This weed, closely related to oilseed-rape, contaminates the crop and can lead to a decrease in oil quality, especially in varieties with low contents of glucosinolates and erucid acid. The soil persistence of trifluralin can negatively influence crop rotation.

The most complicated and expensive weed control system is used in sugar beet (Table V).

Table V. Weed Control Systems in Sugar Beet

No. of applications 1	2	3	4
Triallate or pyrazon or mixtures + mechanical weeding	Metamitron or pyrazon + mixtures phenmedipham, ethofumesate + mechanical weeding	Metamitron or pyrazon + mixtures phenmedipham ethofumesate fluazifop	Mixtures of metamitron, phenmedipham ethofumesate, lenacil, fluazifop
2 %	16 %	43 %	32 %
Area treated			

Often four applications, in some cases even five, of up to six different herbicides are used, to protect the less competitive sugar beet from weed competition. Up to 6 kg of active ingredients are used to have clean fields without any weed infestation. Despite the efficacy of the system there are also limitations like low flexibility in application, erosion, phytotoxicity, costs, and difficulty in use.

Having now looked at the standard weed control systems in these three crops and their limitations, what would be the properties of an ideal weed control system or an ideal chemical? These properties are listed in table VI.

Table VI. Comparison of Ideal Chemical Weed Control with Glufosinate Properties

Ideal Herbicide	Glufosinate Properties
fully selective (no phytotoxicity to crop)	yes[a]
full control of weeds (dicots, monocots, annuals, perennials)	yes
high flexibility	yes
ease of use	yes
no evolution of resistance	yes
rapid degradation	yes
good toxicological and ecotoxicological profile	yes
fits into integrated crop management	yes
fits into "no tillage" systems	yes
cost effective	yes,in some crops

[a] only in engineered crops

The ideal crop production system is safe for the crop and non target organisms. It does not disturb the soil texture and its microbial activity. It prevents soil erosion even under conditions of high wind velocity and heavy rainfall and it helps to minimize the leaching of nitrate. From this point of view, tillage-based weed control systems and preemergent weed control by residual herbicides are far from the ideal situation.

Reduced tillage practices, combined with the application of selected postemergent herbicides, which are applied only if the weed infestation exceeds a threshold level, would be superior. Such herbicides should have broad herbicidal activity with no toxicity to the crop and other non target organisms. Furthermore, they should have high biodegradability, no accumulation of residues in soil and water, and good toxicological and ecotoxicological profiles of the compounds and their metabolites.

Will Glufosinate in Tolerant Crops meet These Criteria?

Glufosinate, a Non-selective Herbicide Worthwhile to Develop Tolerant Crops? Let us first concentrate on the compound L-phosphinothricin, the active principle of glufosinate. It is an amino acid and part of the tripeptide bialaphos which is produced by the soil microorganisms Streptomyces viridochromogenens (8) and S. hygroscopicus (9). Glufosinate is the chemically synthesized amino acid racemate of phosphinothricin. It is a potent non-selective herbicide (10). It is taken up by leaves after foliar application and controls all weeds, depending on the dose. It fulfills the above mentioned toxicological and ecotoxicological criteria, i. e. rapid biodegradation and no persistance (11,12). The phytotoxicity is due to the inhibition of glutamine synthetase (13), an essential enzyme in plants, which fixes the ammonia coming from nitrite reduction, amino acid degradation, and photorespiration. Despite other disputed theories (14), so far the fast inhibition of photosynthesis by the ammonia, not fixed by the glutamine synthetase, seems to be the next step in the generation of phytotoxicity. The action of glufosinate, like that of PSII inhibitors (e.g. triazines), is slower than that of paraquat, but faster than that of other herbicides that interfere directly with amino acid metabolism (e. g. glyphosate, sulfonylureas). In the soil, glufosinate is rapidly inactivated via transamination of the amino-nitrogen (15).

Since the properties of glufosinate are extraordinary and the only flaw is that it cannot discriminate between weeds and crops, the generation of tolerant crops was regarded as desirable (16). This becomes obvious when we compare the properties of an ideal chemical for weed control with glufosinate (Table VI).
There is a perfect fit, and glufosinate is one of the few herbicides for which the generation of tolerant crops fulfills these criteria.

Improved Post-emergent Weed Control System with Lower Environmental Impact

Mechanisms by Which Crops Can Acquire Tolerance to Glufosinate

During several years of intensive in vitro selection for glufosinate tolerance with plant cell cultures it was not possible to select for glufosinate tolerance levels sufficient for field use (17). Similarly, overexpression of the target enzyme glutamine synthetase in plants did not lead to an agronomically useful level of tolerance (18). However, another approach has proved fruitful. In cooperation with Prof. A. Pühler's group in Bielefeld (Germany), we were able to isolate a gene for an enzyme from one producer-strain of bialaphos (19), which in the microorganism inactivates phosphinothricin by acetylation (20). N-acetyl-phosphinothricin does not bind to the glutamine synthetase and has no herbicidal activity.

By adapting the DNA-sequence of the Streptomyces viridochromogenes gene coding for this phosphinothricin-acetyltransferase (PAT) to the codon usage of plants and after inserting the gene into transformation vectors, a range of dicot crops could be transformed using the Agrobacterium tumefaciens vector system (21). Because the transformants contain the enzyme which detoxifies the compound by acetylation, they can be selected on media containing glufosinate. Plant extracts incubated with radioactive L-phosphinothricin will convert 0.5 mM completely within one hour into the inactive N-acetyl-compound. Plants carrying the gene and expressing the enzyme can tolerate at least three times the amount of glufosinate necessary for routine weed control.

It is worthwhile to mention that genes conferring resistance to glufosinate can be efficiently used as selectable marker genes, which help to discriminate between transgenic and non-transgenic individuals on the cellular as well as on the plant level. The glufosinate-resistance genes are therefore excellent breeding tools to introduce other genes of agronomic importance into plant cells, breeding lines, and varieties if these additional genes are closely linked to a PAT gene.

In addition to transformed dicot species, a maize protoplast transformation system for corn has beed developed at Hoechst, and fertile transgenic corn plants have been regenerated. Based on an embryogenic corn cell suspension developed in cooperation with BRC-Szeged (22), corn protoplasts could be transformed with naked DNA containing the synthetic PAT gene under the control of strong promoters. Tolerant calli were selected on glufosinate-containing medium and regenerated to plantlets, which were sprayed with glufosinate after potting. These plants were completely tolerant to 2 kg a.i. /ha (23), about three to five times the amount necessary for efficient weed control.

Using this reliable and effective protoplast transformation and regeneration protocol a large number of transformants could be generated. The majority of the transgenic maize plants are fertile, and the introduced gene is stably expressed over six generations. Beside the synthetic gene from our laboratory, the bialaphos resistance (BAR) gene from Streptomyces hygroscopicus (7) and the natural phosphinothricin-acetyl-transferase (PAT) gene from Streptomyces viridochromogenes (24) have been successfully used in gene transfer experiments. More than a dozen dicot crops and the monocot crops corn, rice and wheat, have now been successfully transformed by several laboratories.

Field Use of Glufosinate-Tolerant Crops But the gene transfer itself is only the starting point for a time consuming development of a product including the breeding of tolerant varieties and the agronomic development of a new weed control system based on glufosinate. In the last few years in several crops, (e.g. soybean, rape seed, corn, and others) the level of glufosinate tolerance was evaluated in the field. The focus has recently been and will be on the evaluation of the amount of glufosinate necessary in a few crops namely in sugar beet, rape seed and corn to efficiently control the weeds. Based on our results, we expect that the dose range listed in table VII will provide good weed control. Even if the higher amounts are necessary, there will be a substantial reduction of totally applied herbicide.

Table VII. The Expected Rate of Glufosinate Required to Achieve Good Weed Control, Compared to the Herbicide Rates most Commercially Used for Weed Control in Each Crop

Crop	Glufosinate	Standard
	- (kg a.i./ha) -	
corn	0.4 - 1	3 - 3
rape seed	0.4 - 0.75	1.5 - 3
sugar beet	0.6 - 1.5	2 - 6

In a soybean trial the transgenic plants showed a sufficient tolerance at 2 kg/ha, and even at 0.5 kg a.i./ha the weeds could be controlled efficiently. In Canadian trials, a transgenic canola line tolerated up to 2 kg/ha and weed control was excellent at 0.75 kg a.i./ha (25). There are indications that weeds can be controlled sufficiently with 0.4 kg a. i./ha. Similarly in corn, transgenic plants tolerated 2 kg a.i./ha without any symptoms and 0.4 kg a.i./ha gave good weed control.

In trials with transgenic canola neither the transformation event itself nor the glufosinate-treatment of the transgenic crop reduced yield. In backcrossing programs using transgenic corn, no pleiotropic effect of the introduced genes could be observed (17). In the coming years all agronomic parameters of this weed control system in crops will be carefully investigated. Besides these parameters, we will study other factors such as the prevention of erosion by forming a layer of mulch from killed weeds. This was clearly documented in an early tobacco trial. After killing the weeds, which almost covered the crop, a dense mulch, covering the soil, was formed (26). This soil mulch could prevent erosion and also help maintain soil moisture.

Based on this effect, one could easily design of no tillage or intercroping systems into which the final crop is seeded, and after emergence, glufosinate is sprayed to kill weeds or cover crops to form a protective mulch. Another aspect which will be important as well, is that through the creation of herbicide tolerant crops, herbicides with unique modes of action can be introduced into agriculture and can become a part of a herbicide rotation system. This may be necessary because use of some of the herbicides developed during the last decade like sulfonylureas, imidazolinones, and grass herbicides is jeopardized by the occurence of spontaneous mutations in weed populations which have already led to herbicide-resistant weeds. Strategies to avoid the evolution of more herbicide-resistant weeds and to slow down their spread have been developed (26,27). Herbicide combinations and herbicide rotations are two effective ways to minimize the risk of herbicide-resistant weeds. Glufosinate as well as glyphosate are two herbicides with unique modes of action, and in both cases no resistant mutant weeds have been found in areas where these compounds have been used repeatedly. Therefore they will be very helpful building blocks in programs to prevent evolution of herbicide-resistant weeds.

In conclusion, we believe that the existing weed-control systems, their ecological limitations, need viable alternatives. These should employ post-emergent, foliar herbicides because they will have lesser impact on the environment. Glufosinate, due to its many desirable properties, will be one of these herbicides, and a system of glufosinate in glufosinate-tolerant crops can be superior to many current weed control systems. If such a system is accepted, the total amount of herbicides applied will decrease, and some of the problems attached to the current herbicide treatment will diminish.

Literature Cited

1. Fraley,T.; Rogers,S.G.; Horsch,R.B.; Kishore,G.M.; Beachy,R.N.; Tumer,N.N. in "Chromosome structure and function. Impact of new concepts. Gustavson,J.P., Appels,R. Eds. Plenum Press New York **1988** p.283-298.
2. Eckes,P.; Donn,G.; Wengenmayer,F. Angew. Chem.**1987**, 99, 392-412.
3. Vaeck,M.; Reynaerts,A.; Höfte,H.; Jansens,S.; De Beuckeleer,M.; Dean,C.; Zabeau,M.; Van Montagu,M.; Leemans,J. Nature **1987**, 328, 33-37.
4. Logemann,J.; Jach,G.; Tommerup,H.; Mundy,J.; Schell,J. Biotechnology **1992**, 10, 305-308.
5. Beachy,R.N.; Loesch-Fries,S.; Tumer,E. Annu.Rev.Phytopathol. **1990**, 28, 451-474.
6. Comai,L.; Facciotti,D.; Hiatt,W.R.; Thompson,G.; Rose,R.E.; Stalker,D.M. Nature **1985**, 317, 741-744.
7. De Block,M.; Botterman,J.; Vandewiele,M.; Dockx,J.; Thoen,C.; Gossele,V.; Movva,N.R.; Thompson,C.; Van Montagu,M.; Leemans,J. Embo J. **1987**, 9, 2513-2518.
8. Bayer,E.; Gugel,K.H.; Hägele,K.; Hagemaier,H.; Jessipow,S.; König,W.A.; Zähner,H. Helv.Chim.Acta **1972**, 55, 224-239.
9. Ogawa,Y.; Tsuruoka,T.; Inouye,S.; Niida,T. Sci.Rep.Meji Seika **1973**, 13, 42-48.
10. Schwerdtle,F.; Bieringer,H.; Finke,M. Z. Pflanzenkrankh. Pflanzenschutz **1981**, SH 9, 431-440.
11. Dorn,E.; Görlitz,G.; Heusel,R.; Stumpf,K. Z.Pflanzenkrankh. Pflanzenschutz **1992**, SH 13, 459-468.
12. Smith,A.E.; Agric. Food Chem. **1989**, 37, 267-271.
13. Leason,M.; Cunliffe,D.; Parin,D.; Lea,P.J.; Mifflin,B.J. Biochemistry **1982**, 21, 855-857,
14. Saver,H.; Nill,A.; Rühle,N. Z. Naturforsch. **1987**, 42c, 270-278.

15. Bartsch,K.; Tebbe,C.C. Appl. Env. Microbiol. **1989**, 55, 711-716
16. Donn,G.; Tischer,E.; Smith,J.A.; Goodman,H.M. J. Mol. Appl. Genet. **1984**, 2, 621-635.
17. Donn,G.; Eckes,P. Z. Pfl. Krankh. Pfl. Schutz **1992**, SH XIII, 499-504.
18. Eckes,P.; Schmitt,P.; Daub,W.; Wengenmayer,F. Molec. Gen. Genet. **1989**, 217, 263-268.
19. Strauch,E.; Wohlleben,W.; Pühler,A. Gene **1988**, 63, 65-74.
20. Imai,S.; Seto,H.; Sasaki,T.; Tsuruoka,T.; Ogawa,H.; Satoh,A.; Inouye,S.; Niida,T.; Otake,N. J. Antibiot. **1985**, 38, 687-690.
21. Donn,G.; Dirks,R.; Eckes,P.; Uijtewaal,B. Abstract VIIth International Congress on Plant Tissue and Cell Culture **1990**, A5-21, p. 176.
22. Morocz,S.; Donn,G.; Nemeth,I.; Dudits,D. Theor. Appl. Genet. **1990**, 80, 721-726.
23. Donn,G.; Nilges,M.; Morocz,S. Abstracts VIIth International Congress on Plant Tissue and Cell Culture **1990**, A2-38, p. 53.
24. Wohlleben,W.; Arnold,W.; Broer,I.; Hillemann,D.; Strauch,E.; Pühler,A. Gene **1988**, 70, 25.37.
25. Oelck,M.M.; Phan,C.V.; Eckes,P.; Donn,G.; Rakau,G.; Keller,W. GCIRC; International Rape seed congress, Saskatoon **1991**, Proceedings Vol.1, A42
26. Duke,S.O.; Christy,A.L.; Hess,F.D.; Holt,J.S. CAST-Report No: 991-1, 1-47.
27. Gressel,J.; Segel,L.A. Amer. Chem. Soc. Symp. Ser. **1990**, 421, 430-458.

RECEIVED August 17, 1992

American Chemical
Society Library
1155 16th St. N. W.
Washington, D. C. 20036

Chapter 4

Synergizing Pesticides To Reduce Use Rates

Jonathan Gressel

Plant Genetics, Weizmann Institute of Science, Rehovot, IL 76100, Israel

There is great need to reduce pesticide inputs to save costs, reduce environmental loads, and lower the selection pressures resulting in rapid evolution of resistance. This can often be done by judiciously chosen mixtures; with synergism given the economic definition of a mixture that gives more cost-effective pest control than the sum of the separate components. Pesticides are often used alone at high rates to control the most recalcitrant pests in the infestation spectrum, where a low rate of a second pesticide can be complimentary and even synergistic. Herbicide mixtures based on field weed spectra matched to the best available compounds are more prevalent in Europe than in the U.S.A. High use rates are often required to overcome endogenous degradation pathways in certain pests, and inhibitors of the offending pathways can be biorationally chosen to synergistically lower rates. This requires a better knowledge of the reasons for the lack-of-action of pesticides in target pests, an area insufficiently researched There are already compounds known that enhance pesticide penetration, that block pesticide degradation by monooxygenases, glutathione transferases, as well as compounds that block detoxification of the active oxygen species generated by many herbicides.

Pesticides have been heavily used as inexpensive low risk 'insurance policies' against pest attack, often whether needed or not. This is especially the case with spring-applied pre-emergence herbicides, the largest use of pesticides in agriculture. They are applied before the spectrum and intensity of weed infestation is known. These herbicides, even when tightly bound to soil, run off attached to silt after heavy spring rains. This has great potential ecological impact.

Many pesticides are used at the highest allowable rates to control the hardest to control pests in a spectrum, exerting the highest possible selection pressures, with concomitant evolution of resistance problems. It is this variation in rates of pesticide to control a spectrum of pests that calls for the development of synergists, based on an understanding of pesticide action and metabolism. The range of concentrations required to inhibit the target site of a pesticide *in vitro* in a variety of species is usually quite narrow; far narrower than the rates required to control the same species in the field, which can vary over a few orders of magnitude. For a pesticide to kill the pest, it must remain at the target site for some time; if the pesticide or toxic compounds generated by it are degraded too rapidly, more pesticide is needed. The prevention of such degradation is a task for synergists; finding such synergists is assisted by understanding what happens to the pesticide in the target pest. If such synergists are successful, it will be

0097–6156/93/0524–0048$06.00/0

possible to lower the levels of pesticides to those required to control the easiest-to-kill species. With residual compounds, more pesticide will be needed to provide the residual effects. Unfortunately, far more is known about pesticide metabolism in crops (ascertained for registration) than in target pests.

This review is mainly devoted to metabolic synergies. Most examples are with herbicides in weeds, as herbicides constitute *ca.* 60% of pesticides used. It is an update of earlier reviews (*1, 2*), to which the reader is referred for a more comprehensive coverage. The farmer has a simple definition of our topic; a mixture is $ynergistic when it results in more net profit than the sum of profits of each component used separately. The patent literature has other definitions (*3*), which are not always the same as the scientific literature (*1, 2, 4-7*), and various methodologies for bioassay and evaluation have been recently summarized (*8*).

Synergistic mixtures can be made without knowing the modes of degradation of pesticides; a good knowledge of the pest spectrum facilitates mixing pesticides known to control each pest at the lowest concentrations. British wheat farmers seem to be adept at doing this, as they usually use mixtures of 2 to 4 herbicides at specially chosen rates (*9*), while their U.S. counterparts use one or at most two, usually at maximum 'label' rates (*10*).

Various additives such as oils, surfactants, and simple compounds such as ammonium sulfate enhance the penetration of pesticides into their targets, and meet both the definition of synergist and $ynergist, but will not be further discussed, despite their field performance and importance.

Some synergistic mixtures are empirically derived and not explainable on precise metabolic grounds; e.g. two herbicide groups that inhibit two different amino acid biosynthesis pathways are synergistic (*11*). It is not clear why two inhibitors of photosystem II that interact ostensibly at the same binding domain should be mutually synergistic. Yet, at the whole plant level both metamitron (a triazinone) and chloridazon (pyrazon) (a pyridazinone) were both synergistic with lenacil (a uracil) on oats (*12*). The differences may be due to a mutual interference in metabolism or due to effects on secondary sites by one of the herbicides. Other metabolic synergies can be indirect; i.e. inhibiting pesticide-degrading microorganisms in "problem soils" (*13*). Such compounds *are* synergists, in the terms of this review, but will not be further discussed as they do not prevent degradation in the pest.

Metabolic Synergists

Metabolic synergists are compounds that substantially increase the toxicity of a pesticide more than additively at the doses applied, by interfering with the pests metabolism. They inhibit the enzymes that detoxify either pesticides or the toxic products resulting from the interaction between the pesticide and the target. They have great potential utility in suppressing both natural and recently evolved metabolic resistances to pesticides.

Synergists can decrease the levels of pesticide needed to control resistant as well as susceptible pests by lowering constitutive levels of the pesticide-degrading enzymes in both biotypes. For this reason they can become of increasing importance in lowering pesticide use levels as well as in pest resistance management. The evolution of resistance can also be prevented by synergists as a proactive treatment. This is because the synergist may allow near-equal control of resistant and susceptible biotypes, and the lower pesticide levels lead to lower selection pressure.

Metabolic synergists have great research value. By applying the pesticide with a synergist, it is possible to study the intrinsic toxicity of the pesticide *in vivo* in the absence of detoxification. Synergists can also be used to explore resistance mechanisms. Any resistance that can be overcome by a specific synergistic enzyme-inhibitor is likely to be caused by the evolution of high levels of the inhibited enzyme. Experiments showing pesticide LD_{50}s with and without a synergist allow computation of "synergistic ratios". These ratios indicate the role of suppression of pesticide metabolism in the pest (*14, 15*). It is then necessary to perform *in vitro* metabolism and enzyme-biochemistry studies to verify the effects on metabolism. If a synergist does not substantially reverse a major resistance, resistance at the molecular target site of the pesticide may be involved.

Selectivity of Synergists. Pesticides are often degraded by the same mechanism in crop and pest. If a synergist prevents degradation in both, it can lead to phytotoxicity or unwanted pesticide residues. This often happens when different classes of pesticides are used; the azole fungicide prochloraz inhibits *in vitro* degradation of the herbicide chlorotoluron (*16*). There were many field reports that terbufos and other organophosphate insecticides used in corn cause phytotoxicity when used with sulfonylurea and imidazolinone herbicides that are selectively degraded in corn (*17, 18*).

Synergists could be used to control weeds in with crops genetically engineered to have target site herbicide resistance. The synergists would not affect crops but would be synergistic in weeds that metabolize the herbicide as a mechanism of tolerance. Additionally, alternate modes of degradation could allow synergist use. For example; pyridate is degraded in most crops and some weeds by a monooxygenase system, and by a rare *N*-glucosyl transferase in peanuts (*19*). The herbicide could be used in peanuts and in other crops engineered with the peanut gene along with a monooxygenase-inhibiting synergist.

The ideal case of synergy was recently found with fenchlorazole-ethyl. This compound is usually used to "protect" wheat and barley from damage by the herbicide fenoxaprop-ethyl, by stimulating herbicide degradation. It was fortuitously found that fenchlorazole-ethyl has no effect on degradation of the herbicide in crab grass (*Digitaria* sp.) (*20*), but the mixture is synergistic in controlling of *Setaria* and *Echinochloa* (*21*).

Limitations to Metabolic Synergists. Not all pesticides can be synergized in all intransigent pests. There must be an active detoxification pathway that can be elucidated and a suppressant must be found. For example only Graminae are affected by the herbicides inhibiting acetyl-CoA-carboxylase. Non-graminaceous species are resistant due to differences in the target enzyme; there is a >100 fold difference in K_i (*22, 23*). A synergist can be developed only if there is differential detoxification. Thus, only the weeds that evolved detoxifying mechanisms to this group of herbicides can be synergized (*24-27*). More than 80 weed species evolved a modified target site and do not bind triazine herbicides (*28*), and these cannot be synergized. Synergists control *Abutilon theophrasti* (*29*) and *Amaranthus* (*30*), species that evolved a biochemical mechanism to detoxify atrazine.

Insecticides are often hard to synergize, as insects often evolve elevated levels of more than one metabolic pathway that degrades the insecticide.

The cause of lack of control of a given species need not be due to detoxification or lack of target, it could be due to having alternative redundant pathways. Higher organisms, often have more than one mechanism to achieve resistance. They have nuclear and extranuclear genomes encoding enzyme families containing discrete isozymes, alternative pathways, etc. A pesticide may not control the pests having the alternative mechanism. This again accentuates the need to know why the pest is not controlled before setting out to find a synergist. In this last case, the synergist can be a compound inhibiting the alternative pathway in the pest that is not found in the crop.

Pesticides that must be activated to a toxic moiety cannot be synergized if the activating enzyme system is lacking. Many pesticides are systemic and must be translocated to their site of action and may be degraded along the way. There are at least two ways to synergize a translocated pesticide: (a) by inhibiting detoxification; (b) by enhancing the rate of translocation. There are compounds known to decrease translocation rates (especially auxins), but there are a few reports of compounds that funtionally enhance total translocation, possibly acting by enhancing penetration.

The greatest limitations to synergists at present are: (a) our insufficient knowledge about the pathways that need synergy; (b) Our insufficient quest for synergists; and (c) the lack of sufficient efficacy of the present synergists. The one herbicide synergist that was released for agricultural use (tridiphane) has been withdrawn from the market due to lack of consistent efficacy. The only commercial insecticide synergist, piperonyl butoxide is used at many-fold higher rates than the pyrethroid insecticides it synergizes, and is used only in resistance management in agriculture, or where there is no cost effective alternative.

SYNERGIZEABLE SYSTEMS

Synergists Inhibiting Glutathione Transferases. The degradation of many xenobiotics is initiated by glutathione-transferases that conjugate the pesticide to glutathione (glutamyl-glycyl-cysteine) or homoglutathione (glutamyl-alanyl- cysteine) (*31*). "Glutathione" will be used loosely for both tripeptides. At least three glutathione transferases are present in corn, each with its own herbicide substrate specificities (*32*). Corn seems to have a very high activity of this enzyme system, especially the glutathione transferases conjugating atrazine and chloroacetamide herbicides (*33, 34*). The genes for the various isoenzymes have been isolated, cloned and sequenced (*35-37*). Glutathione is irreversibly conjugated by glutathione transferases to herbicides such as atrazine. As glutathione is used up stoichiometrically in pesticide metabolism, compounds that inhibit the biosynthesis of glutathione (*38*), or the action of the transferase should also act as synergists.

Glutathione transferases are prevalent in many panicoid grasses such as *Setaria* spp. and *Panicum* spp. These species do not degrade atrazine as rapidly as corn, allowing selectivity, but high rates of atrazine are required to overcome the weeds' glutathione transferases. Such high rates can increase ground and runoff water pollution, while supplying the high selection pressure for selecting for resistance, and leave residues precluding rotation with sensitive crops. *Abutilon theophrasti* has evolved atrazine resistance by biochemically "mimicking" corn; one gene controls an elevated glutathione transferase level (*29*).

Synergy by inhibiting glutathione transferase conjugation was fortuitously found when tridiphane, a grass herbicide, was checked for compatibility with atrazine (Table I). Tridiphane alone had only limited activity but the tridiphane-atrazine mixture proved highly synergistic (*43, 44*). Indirect evidence showed a blockage of atrazine catabolism. Tridiphane did not directly inhibit the activity of purified glutathione transferase. A glutathione transferase was found that irreversibly conjugates tridiphane with glutathione, forming a glutathione transferase-inhibiting complex (*41, 46*). This conjugate is a far more potent inhibitor of the glutathione transferase than tridiphane. The tridiphane-glutathione transferase complex was able to inhibit the glutathione transferases that degrade propachlor and fluorodifen, as well as atrazine and other xenobiotics (*47*). Similar inhibitors of mammalian glutathione transferases have been found that similarly act by conjugation and inhibit glutathione transferase (*48*).

Tridiphane synergizes atrazine, killing weeds in corn-fields, yet the corn is unaffected. This is surprising, as the tridiphane-glutathione conjugate inhibits the corn glutathione transferase that degrades atrazine. This inhibition of glutathione transferase activity is weaker in corn than in *Setaria*. A kinetic analysis showed that the tridiphane-glutathione conjugate is a competitive inhibitor of glutathione, binding to glutathione transferase with a four-fold higher affinity for the enzyme from *Setaria* than the corn enzyme (*41*). The *in vitro* data have been verified in whole plant experiments that also show tridiphane-glutathione conjugation (*46, 47*). The tridiphane-glutathione conjugate concentrations *in vivo* are high enough to explain the inhibition of glutathione transferase in *Setaria,* but are insufficient to explain why tridiphane does not kill corn. Young corn leaves have six times more glutathione than *Setaria* (*41*), which could partially explain the difference, but older corn and *Setaria* leaves have the same concentration. The selectivity between corn and panicoid weeds is thus probably a complex series of events. These include the ability of corn to degrade much of the atrazine before tridiphane forms an inhibitory conjugate, whereas the grasses do not degrade atrazine as quickly. The residual atrazine is not toxic because corn remains

Table I. Metabolic Synergists That Act By Inhibiting Glutathione Transferases

Pesticide	Synergist	Target Organism/ Enzyme Source	ref.
	-in vitro-		
organophosphates (i)[a]	var. saligenin cyclic phosphates	equine and fly	*39, 40*
atrazine(h)[a]	tridiphane	var.plant and equine	*41,*
diazinon (i)	tridiphane	fly	*42*
	-in situ-		
EPTC(h), alachlor(h),	tridiphane[b]	*Panicum* sp.	*43*
atrazine (h)	tridiphane[b]	many grasses	*44, 45*

[a] i= insecticide; h= herbicide

[b] Tridiphane was able to synergize a large number of herbicides in whole plant systems including metribuzin (especially on *Matricaria* and *Abutilon),* diflufenican (7 spp.), propanil (especially *Echinochloa*) among many (*45*), but the reasons may be due to an inhibition of other enzymes in some of these cases.

alive by virtue of having more glutathione and less tridiphane-glutathione conjugate than the grasses, with less activity and faster degradation of the conjugate. Thus, tridiphane must first be conjugated in the target organism. The glutathione-transferase is then inhibited by the tridiphane-glutathione conjugate complex, which must be stable for tridiphane to synergize atrazine or other pesticides that are conjugated to glutathione.

The effect of tridiphane is not limited to synergizing atrazine in grasses. Metribuzin, diflufenican and propanil were synergized in a wide variety of weeds by tridiphane (*45*). Tridiphane also prevents degradation of EPTC and alachlor in corn, an undesirable synergy (*45*). The modes of these synergies have not been elucidated. Tridiphane surprisingly also synergized chlorotoluron, which is not conjugated by glutathione (*49*).

Tridiphane also synergized the insecticide diazinon against houseflies (*42*). Tridiphane had a twenty-fold greater rate of conjugate formation with glutathione than diazinon, and diazinon does not seem to compete with this step. *N*-ethymaleimide (*50*) and saligenin cyclic phosphates (*40*) synergize organophosphate insecticides by inhibiting glutathione transferase.

Synergists Inhibiting Monooxygenase Activities. Many plant species possess an array of enzymes capable of oxidizing pesticides. They can confer crop vs. pest selectivity, as well as control the differences in rates required to control different pest species. The oxidation step introduces a single oxygen from molecular oxygen atom into the pesticide molecule, with the other oxygen atom forming water. There is direct evidence in many cases that this monooxygenation is performed by NADPH-dependent cytochrome P_{450} mixed function oxidases (cyt-P_{450}) (*51*). These cyt P_{450} monooxygenases are bound to plant microsomal membranes. These enzymes are soluble in bacterial pesticide-degrading systems (*51*). There is good evidence for membrane bound cyt-P_{450} mediated pesticide degradations in insects, weeds, and fungi.

Recently, a new type of herbicide resistance has evolved, which is ascribed to cyt-P_{450} systems (*52*). Two grass-killing herbicides, diclofop-methyl and chlorotoluron have selected for resistant populations of *Lolium rigidum* and *Alopecurus myosuroides,* respectively (*24-27, 52*). These new weed biotypes are also resistant to all other selective herbicides for wheat that previously controlled wild type weed populations. These include inhibitors of acetolactate synthase, acetyl CoA carboxylase, photosystem II, and tubulin polymerization. A metabolic cross resistance involving a single enzyme system probably evolved in these weeds. The weeds that evolved this resistance may have evolved the predominant mechanism of wheat for degrading herbicides, i.e. by oxidation (*52*). The available literature suggests that wheat detoxifies the selective herbicides used in weed control for this crop by oxidation (*52*). Wheat oxidizes these herbicides at a large number of sites, both on the rings and side chains of the herbicides (*53, 54*). Inhibitor studies suggest that more than one polysubstrate monooxygenases are found in plants. The inhibition of flumetsulam metabolism to its difluorophenyl hydroxylated product was greater by paclobutrazol, and the inhibition to the pyrimidine hydroxylated product was greater by piperonyl-butoxide (*55*). There is yet no information how such systems are genetically controlled in resistant weeds. Another *Lolium rigidum* resistant biotype is capable of *N*-dealkylating atrazine and is not capable of hydroxylating wheat-selective herbicides (*56*). This is of less importance in our context, as monooxygenase inhibitors that selectively synergize particular pesticides are needed (Table II).

Piperonyl butoxide is used to synergistically inhibit the oxidation of insecticides, especially with the very expensive pyrethroids (*77*), especially with pyrethoid-resistant insects, by blocking the degradation of the insecticides. Piperonyl butoxide can be used to synergize herbicides in corn (*74*), but no evidence was presented that it acts by specifically inhibiting plant monooxygenases. Piperonyl butoxide partially suppressed the evolved cross-resistances to diclofop-methyl and chlorsulfuron in *Lolium* (*75*).

Aminobenzotriazole is a well known monooxygenase inhibitor in mammalian systems. It is also an inhibitor of chlorotoluron hydroxylation in wheat, but impotent as a synergist on *Veronica persica*, which *N*-dealkylates this herbicide (*69*). This is the antithesis of what is desired from a herbicide synergist, as the crop but not the weed was controlled. Different types of monooxygenases may be involved in pesticide degradations and they can be differentially synergized. This indicates that nuances are present and that it may be possible to find a monooxygenase inhibitor specifically affecting weeds and not certain crops, as was found with tridiphane. Synergism by aminobenzotriazole has often been used as proof for action of cyt-P_{450}s. This can be highly speculatory as this compound also inhibits sulfoxidases that do not contain cytochrome P_{450} (*78*).

Fungicide research has centered on finding fungicidal but non-phytotoxic inhibitors of sterol biosynthesis (*79, 80*). These azole derivatives affect late monooxygenase steps leading to ergosterol. Some of them are plant growth retardants, blocking a monooxygenase step (kaurene oxidase) in gibberellic acid biosynthesis. These can block other plant terpenoid, as well as sterol biosyntheses (*80*). Tetcyclasis, a known kaurene oxidase inhibitor, was tested for its ability to prevent herbicide degradation in corn and cotton, where it was a hundred times more active than aminobenzotriazole (*80*). Tetcyclacis was also highly active in preventing the degradation of the photosystem II-inhibiting herbicide bentazon in both wheat and soybean. Tetcyclasis is not likely to be used in the field as it is photolabile. Other kaurene oxidase inhibitors such as paclobutrazol also synergize herbicides used in wheat by inhibiting their degradation by monooxygenases.

The most peculiar synergism of monooxygenase degraded herbicides is by tridiphane, the suicide inhibitor of glutathione transferases, described in the previous section. Tridiphane synergized isoproturon in both susceptible and resistant *Alopecurus* (*49*). Tridiphane was also found to directly affect microsomal monooxygenases (*59*).

Many herbicide safeners protect crops from damage by stimulating glutathione transferase production. Some act as synergists, preventing the metabolism of herbicides and the insecticide diazinon by monooxygenases in plant microsomal systems (*81*). This effect is expected by analogy with known mammalian stimulators of glutathione transferase. Their stimulation is actually indirect, occurring only after inhibition of monooxygenases (*82*).

A few studies have shown that there are many species differences in effects of monooxygenase inhibitors, using the highly specific monooxygenases catalyzing synthetic pathways in plants. The same specific enzyme is inhibited in some species and not in others. Some inhibitors inhibit one enzyme and others inhibit many (*55, 64, 83*), suggesting that there should be selective synergists that specifically syner-

Table II. Synergists That Act by Inhibiting Cyt-P-450 Monooxygenases

Pesticide (process[a])	Synergist[b]	Species	(ref.)
	Microsomal Systems		
Bentazon (hydroxln)	Tet	corn	*57*
	unstated	sorghum	*58*
Metolachlor (hydroxln)	Tet, PBO, tri	sorghum	*59*
Chlorotoluron (hydroxln)	ABT,Mdn	wheat	*60*
Diclofop (hydroxln)	Tet>Pac>Pro	corn	*61*
	Tet	wheat	*53*
Triasulfuron (hydroxln)	PBO, Tet	wheat/corn	*54, 62*
Furanocoumarins (synth)	Tet, Anc	*Amni,* others	*63, 64*
Flumetsulam (hydroxln)	Tet, PBO, Pac	corn	*55*
	Cell cultures		
Chlorotoluron (hydroxln)	ABT,DPP,Tet	cotton/corn	*66*
	prochloraz	*Alopecurus,* wheat	*16*
Furanocoumarins (synth)	Tet, Anc	*Amni,* others	*63,64*
	Intact -Tissues		
MCPA (metab.)	ABT	potato	*65*
Chlorotoluron (hydroxln)		corn,cotton,	*66*
		wheat	*67, 68*
		Bromus, Alopecurus	*69, 70*
Bentazon (hydroxln)	Mtp>B1>BO>Tet	soybean	*71*
	Tet, B1	*Amaranthus,* etc	*72*
Primisulfuron	Tet>ABT	*Echinochloa*	*73*
Triazines	PBO	corn	*74*
Isoproturon, others	Tri	*Alopecurus*	*49*
Diclofop-methyl	PBO	*Lolium*	*75*
Difenzoquat[c]	Pro	*Avena*	*76*

[a] Abbreviations: hydroxln, hydroxylation; synth., synthesis; metab, unspecified metabolism.

[b] Abbreviations: ABT, aminobenzotriazole; Anc, ancymidol; B0, BAS110; B1 BAS111; DPP, 3-(2,4-dichlorophenoxy-1-propyne); Mdn, menadione; Mtp, metyrapone; Pac, paclobutrazol; PBO, piperonyl butoxide; Pro, propiconazole; Tet, tetcyclacis; tri, tridiphane.

[c] Not clear if working via cyt. P-450.

gize herbicides in hard to control weeds and not in crops. These compounds may already may already exist but have never been screened for synergism: industry has synthesized tens of thousands of azole derivatives and screened them for fungicidal (anti-ergosterol) and PGR (anti-gibberellic acid) activity. These should be rechecked for synergy with "favorite" herbicides on specific weeds.

Synergists Suppressing the Oxidant Detoxification Pathway. Many herbicides kill plants by photogeneration of active oxygen species. The triazines, phenylureas, uracils and some of the pyridazinones block electron transport at the reducing side of photosystem II of photosynthesis just before plastoquinone reduction (*84*). Some phenolic herbicides act at a nearby site on photosystem II. The bipyridilium herbicides drain electrons from photosystem I, probably from ferredoxin (*85*). Diverse herbicide chemistries including the nitrodiphenylethers cause plants to accumulate the photodynamic pigment protoporphyrin IX by blocking the enzyme that oxidizes a precursor to protoporphyrin IX (*86, 87*). This induces an over-production of the precursor and its spontaneous oxidation to protoporphyrin.

It is not fully clear which active oxygen species is /are generated first with oxidant-generating herbicides. Membrane lipoxidation ensues when more active oxygen is generated than can be coped with by the endogenous detoxification system. This results in water loss, a general breakdown in the membrane-bound electron transport systems, and the transfer of solar energy to a variety of active oxygen species that can be confused with the first type generated. Chlorophyll radical, singlet oxygen, superoxide, hydroxyl radicals, etc. can be produced. This represents a self- amplifying toxic chain reaction of generation of active oxygen species; membrane lipoxidation and water leakage lead to the rapid desiccation caused by these herbicides. The stronger the light, the faster this chain reaction. Certain environmental xenobiotics (SO_2, O_3, NO_x) and some fungus-produced toxins such as cercosporin (*88*), have similar effects. There are caveats in the literature stating that it is unlikely that singlet oxygen can be produced in leaves, where there are high levels of reductants such as NADPH, reduced glutathione, and ascorbate (*89*).

Plants have endogenous active oxygen detoxification systems, which probably evolved together with photosynthesis and aerobic respiration to cope with energy "leakages" from the electron transfer chains. They include superoxide dismutase and the ability to produce and recycle oxyradical quenching agents such as glutathione and ascorbate. Plants also produce carotenes (*90*) and α-tocopherol (*91*), which quench these active species. More radicals must be produced than can be quenched by these systems before the herbicide is dissipated for a phytotoxic response. Chloroplast-produced active species such as singlet oxygen and superoxide have very short diffusion distances and must be quenched before they reach membranes if the plant is to remain undamaged. The chloroplasts have their own concerted antioxidant defense mechanisms including the slowly recyclable carotenes, as well as an enzymatic pathway for oxygen detoxification.

The oxygen detoxification pathway uses a series of enzymes while recycling ascorbate and glutathione. It has been put in context, especially by Halliwell (*92*) and Asada (*93*). The first enzyme is superoxide dismutase, which dismutates superoxide to hydrogen peroxide. Hydrogen peroxide is less reactive than superoxide and can diffuse to other cellular compartments, but it is still potentially destructive. Hydrogen peroxide can react with ferrous iron and form hydroxyl radicals (the Fenton reaction). Hydroxyl radicals are extremely damaging, more than superoxide. If hydrogen peroxide is formed from superoxide, excess superoxide can react with the ferric ions, formed by the Fenton reaction, re-reducing them to ferrous ions, making them available to form more hydroxyl radicals. The Fenton reaction coupled with the iron recycling reaction is termed the Haber-Weiss reactions. Plants must get quickly be rid of hydrogen peroxide to survive, to prevent this cycled production of radicals.

Chloroplasts do not contain catalases, that directly degrade hydrogen peroxide but do contain ascorbate peroxidase and ascorbate, which can adequately compete with the Fenton reaction to remove the potentially destructive peroxide. The dehydroascorbate produced is recycled to ascorbate by glutathione, either spontaneously or by a dehydroascorbate reductase. The oxidized glutathione is recycled to glutathione by glutathione reductase, utilizing NADPH. Some NADPH must be produced to support this reaction. This system will also recycle both the glutathione and ascorbate used to quench singlet oxygen and other radicals.

The Halliwell-Asada pathway enzymes and reactants have been found in leaves of all species where sought. There is considerable evidence that elevated levels of the Halliwell-Asada pathway enzymes are at least partially responsible for increased tolerance to some active oxygen generating herbicides, keeping the plant alive until the herbicides can be dissipated. Kinetic data show that paraquat sprayed on leaves of the paraquat-resistant biotype of *Conyza bonariensis* rapidly but transiently inhibits photosynthesis (*94*) until paraquat is dissipated. The three major enzymes in the Halliwell-Asada active oxygen degradation pathway are constitutively elevated in chloroplasts of the resistant *Conyza* (*95*). Resistance was dominantly inherited under the control of a single gene. The levels of the three enzymes were elevated in the F_1 generation and high enzyme levels segregated with resistance in the F_2 (*95*). The high levels of the Halliwell-Asada pathway enzymes in this biotype conferred some resistance to oxidative stress caused by other herbicides and xenobiotics (*96*).

Blocking the Halliwell-Asada pathway and preventing regeneration of its products decreases tolerance to oxidants. Compounds suppressing the pathway are potential synergists. The plastid superoxide dismutase and ascorbate peroxidase are both copper-containing enzymes. The former contains zinc, and the latter tightly bound iron and thiol groups. The later enzymes of the pathway contain thiol groups as well (*97*). There are few copper-containing enzymes and fewer yet zinc containing enzymes in plants. Chelators of copper and zinc synergized oxidant generating herbicides (*98*) both in *Conyza* (*98*) and in *Solanum* (*99*). Conversely, iron-chelating compounds protected cells from paraquat, presumably by blocking the Haber-Weiss reactions, preventing hydroxyl radical formation. The first successful copper chelator/synergists tested were potent at orders of magnitude lower concentrations in cell cultures and floating leaf discs than on whole plants (*98*). This was thought to be due to some being too hydrophobic and others too hydrophilic. Chelators of copper were synthesized with a better hydrophilic / lipophilic balance. Chelators were capable of synergizing oxidant generating herbicides 4 to 10 fold, when they were used in the micromolar range (*100*).

Inhibition of Glycosyl Transferases. Glycosyltransferases conjugate a sugar moiety (usually glucose) directly to a substrate. They thus render lipophillic herbicides highly polar, as well as changing their bulk configuration. Glycosylated herbicides are usually inactive. Two positions of glycosylation are common: *O*-and *N*-glycosylation, to oxygen and nitrogen groups, respectively. *O*-Glycosylation reactions are usually reversible by relatively non-specific β-glucosidases that are common in cells. *O*-Glycosylation of an active pesticide, *temporarily* inactives the compound, which can be reactivated. Xenobiotics are often *O*-glycosylated after an inactivating hydroxylation. Thus, it seems impossible to synergize pesticides at the level of inhibiting *O*-glycosyl transferases, although little is known about the nature and relevance of reactivation of *O*-glycosylation to active compounds. *N*-Glycosylation seems to be an irreversible conjugation reaction. The easiest method to estimate whether a compound is *N*-or *O*-glycosylated is to react the product with a β-glucosidase. The product is probably *N*-glycosylated if it remains uncleaved. There are no leads as yet on inhibitors of *N*-glycosyl transferases, but such compounds could clearly synergize many herbicides.

Esterase Inhibitors. Many insecticides are degraded by esterases, and various esterase inhibitors can synergize such degradation. For example DEF (*s, s, s* tributylphosphorotrithioate) and phenyl saligenin cyclic phosphonate synergized the insecticide diflubenzuron by 2 to 3 fold (*101*), and some pyrethroids were similarly synergized by other phosphorothioates (*102*).

Inhibition of Amidases. The herbicide mefenacet, which is used for *Echinochloa* control in rice was fortuitously found to be synergized by the phosphorodithioate fungicide edifenphos. This was traced to an inhibition of an amidase reaction that cleaves mefenacet, including in *in vitro* experiments (*103*). Luckily, edifenphos does not prevent mefenacet degradation in rice. Propanil detoxification in rice is similarly inhibited by amidase-inhibiting insecticides, as reviewed earlier (*2*).

Synergies for Unclear Reasons. Both picolinic acid-*t*-butyl-amide (PABA, MZH2091) and the herbicide safener dichlormid synergize metribuzin as well as other oxidant generating herbicides with different sites of action (*104, 105*) It was initially thought that this was due to an inhibition of metribuzin deamination, but *in vitro* data did not parallel the *in situ* data (*104*). As these compounds also decreased ascorbate levels, it was thought that they might (indirectly) prevent oxidant scavenging (*105*) Mefluidide similarly enhanced the activity of oxidant generating herbicides with different sites of action (*106*).

Target-Site Resistances. Target-site resistances have appeared due to mutations in herbicide binding sites that allow the pest to continue living. It should be very hard to design a synergist for such situations, except when "negative cross resistance" is known. This is a phenomenon whereby a pesticide controls the resistant type at lower rates than it controls the wild type (*107*). In some cases this is due to enhanced binding to the so-called resistant site. For example, phenolic herbicides and pyridate (photosystem II inhibitors) are far more potent in inhibiting these reactions in triazine-resistant biotypes than sensitive biotypes (*107, 108*). The triazine resistance modification changed the configuration of the receptor protein (the D_1-Q_B binding protein of the photosystem II reaction center) such that it has greater affinity to these herbicides. There will be a synergistic effect if the herbicides with negative cross-resistance change the molecular configuration upon binding in such a manner that triazines will *also* bind.

Synergizing Biocontrol Agents. The use of synergists is not limited to overcoming the defenses to chemical pesticides. Pests evolved methods to fend off "pests of pests" long ago, and we must study these host-parasite relations. We can learn from plants. Insects die more readily from the *Bt* bacteria on some plants than others. This is because some plants produce orthoquinone synergists that enhance toxicity of the *Bt* bacteria (*109*). Mycoherbicidal organisms should be 'easy' to synergize as the weeds employ a series of defenses that can be overcome. For example, the weed *Cassia* synthesizes a specific dihydroxychromone phytoalexin (partly derived from the shikimate pathway) after attack by a specific mycoherbicide (*110*). Sub-phytotoxic doses of glyphosate, a specific inhibitor of the shikimate pathway greatly enhances mycoherbicide infection, i.e. acts as a synergist, without affecting crop selectivity (*111*).

Concluding Remarks

We now know more about the pathways of pesticide degradation than ever before, thanks partly to registration requirements. There are ample model inhibitors of these degradation pathways, to ascertain the potential for synergies. Once a synergy is shown, it is time to optimize the synergist, using the same approaches of QSAR used for pesticides. This must be done initially with target pests and *not* crops; there are too many excellent synergists for crops, but not for weeds.

Often, there are failed pesticides derived from 'discovery' programs based on targeting specific enzymes; the compounds turned out to be 'wimps' lacking sufficient pest toxicity. These compounds are clear candidates for synergistic mixtures, with similar compounds. We expect too much when we suppose that every excellent enzyme inhibitor will kill a pest.

Because of the perceived near double registration costs for synergistic mixtures, industry has shied away from using the cerebral approach in finding and developing synergists, even though the cerebral approach is far more amenable to finding metabolic synergists than for finding pesticides themselves. The intensive political efforts to mandate less pesticide use should logically induce more effort, not yet apparent, in finding synergies. The demands are for brain over brawn, i.e. thoughtful use of pesticide synergies over massive pesticide use. Surely there is a niche for developing metabolic synergists in the thoughful approach.

Acknowledgments

The author thanks many colleagues for providing unpublished findings, and data prior to publication. The author's current research on oxidant tolerance is supported by the Minerva Foundation. The author is the Gilbert de Botton Professor of Plant Science.

Literature Cited

1. Green , J.M.; Bailey, S.B. In *Methods of Applying Herbicides,* Mc Whorter, C.G. .; Gebhardt, G. B.; Eds.; Weed Sci. Soc. Amer: Champaign, 1989 pp. 37-61.
2. Gressel, J. *Rev. Weed Sci.* **1990,** *5,* 49-82.
3. Richer, D.L. *Pestic. Sci.* **1987,** *19,* 309-15.
4. Levy, Y.; Benderly, M.; Cohen, Y.; Gisi, Y.; Bassand, D. *EPPO Bull.* **1986,** *16,* 651-7.
5. Pöch, G. *Arch. Complex Environ. Stud.* **1991,** *3,* 65-74.
6. Altenburger, R.; Bödeker, W.; Faust, M.; Grimme, L.H. *Ecotox. Environ. Safety* **1990,** *20,* 98-114.
7. Bödeker, W.; Altenburger, R.; Faust, M.; Grimme, L.H. *Nach. Deut. Pflanzenschutzd.* **1990,** *42,* 70-8.
8. *Herbicide Bioassay;* Streibig, J.C.; Kudsk, P., Eds.; CRC Press: Boca Raton, FL, 1992 (in press).
9. Sly, J.M.A.; *Survey Report No. 41,* Min. Agric. Food, Fisheries: London, 1986.
10. McMullen, M.P.; Dexter, A.G.; Nalewaja, J.D.; Hamlin, W.; Davison, K. *Pesticide use on major crops in North Dakota.* North Dakota State Univ.: Fargo, 1985.
11. Bocion, P.F. *Eur. Patent Appl.* 1986, EP 234-389.
12. Streibig, J.C. *Weed Res.* **1986,** *2,* 207-214.
13. Pelsy, F.; Leroux, P.; Heslot, H. *Pestic. Biochem Physiol.* **1987,** *27,* 182- 8.
14. Brattsten, J.B.; Metcalf, R.L. *J. Econ. Entomol.* **1970,** *63,* 101-
15. Hollingworth, R.M. In *Insecticide Biochemistry and Physiology,* Wilkinson, C.F., Ed; Plenum: New York, 1976, p. 431-506.
16. Cabanne, F.; Canivenc, M.C.; Gaudry, J.C.; Scalla, R. *Weed Science Society Amer. Abstracts* **1990,** *30,* 283.
17. Diehl, K.E.; Stoller, E.W. *Weed Science Society Amer. Abstracts,* **1992,** *32,* 295.
18. Bauman, T.T.; Owen, M.D.K.; Liebl, R.A. *Weed Science Society Amer. Abstracts,* **1992,** *32,* 44.
19. Zohner, A. In *Brit. Crop Prot. Conf. - Weeds;* 1987, pp. 1083-90.
20. Yaacoby, T.; Hall, J.C.; Stephenson, G.R. *Pestic. Biochem. Physiol.* **1991,** *41,* 296-304.
21. Stephenson, G.R.; Tal, A.; Vincent, N.A.; Hall, J.C. *Weed Sci. Soc. Amer. Abstracts,* **1992,** *32,* 291.
22. Secor, J.; Cseske, C. *Plant Physiol.* **1988,** *86,* 10-2.
23. Burton, J.L.; Gronwald,; Somers, J.A.; Connally, J.A.; Gegenbach, B.G.; Wyse, D.L. *Biochem. Biophys. Res. Comm.* **1987,** *148,* 1030-8.
24. Gressel, J. *Oxford Surv. Plant Molec. Cell. Biol.* **1988,** *5,* 195-203.
25. Heap, I.M. Ph.D. Thesis, Univ. Adelaide, 1988, 162p.
26. Powles, S.B.;Matthews, J.M. In *Achievements and Developments in Combating Resistance.* Denholm, I.; Devonshire, A.; Hollomon, D., Eds.; Elsevier: London, 1992 (in press).
27. Moss, S.R.; Cussans G.W. In *Herbicide Resistance in Weeds and Crops,* Caseley, J.C.; Cussans, G.W.; Atkin, R.K., Eds.; Butterworths: Oxford, 1991, p. 45-56.
28. LeBaron, H.M.; McFarland, J. In *Managing Resistance to Agrochemicals,* Green, M.B.; LeBaron, H.M.; Moberg, W.K., Eds.; Amer. Chem. Soc..: Washington, 1990, pp. 336-352.
29. Anderson, M.P.; Gronwald, J.W. *Plant Physiol.* **1991,** *96,* 104-9.

30. Beurat, E.; Maigre, D. In *13e Conf. du Columa,* 1986, p. 23-31.
31. Mannervik, B.; Danielson, U.H. *CRC Crit. Rev. Biochemistry.* **1988,** *23*, 283-337.
32. Mozer, T.J.; Tiemeier, D.C.; Jaworski, E.G. *Biochemistry* **1983,** *22*, 1068-72.
33. Shimabukuro, R.H.; Frear, D.S.; Swanson, H.R.; Walsh, W.C. *Plant Physiol.* **1971,** *47*, 10-4.
34. O'Connell, K.M.; Breaux, E.J.; Fraley, R.T. *Plant Physiol.* **1988,** *86,* 359-63.
35. Shah, D.M.; Hironaka, C.M.; Wiegand, R.C.; Harding, E.I.; Krivi, G.G.; Tiemeier, D.C. *Plant Molec. Biol.* **1986,** *6,* 203-11.
36. Wiegand, R.C.; Shah, D.M.; Mozer, T.J.; Harding, E.I.; Diaz-Collier, J.; Saunders, C.; Jaworski, E.G.; Tiemeier, D.C. *Plant Molec. Biol.* **1986,** *7,* 235-43.
37. Timmerman, K.P. *Physiol. Plant.* **1989,** *77,* 465-71.
38. Meister, A. *Science* **1983,** *220,* 473-477.
39. Shiotsuki, T.; Kakimoto, T.; Eto, M. *Pestic. Biochem. Physiol.* **1992,** *42*, 119-127.
40. Shiotsuki, T.; Koiso, A.; Koiso, A.; Eto, M. *J. Pestic. Sci.* **1989,** *14,* 337-44.
41. Lamoureux, G.L.; Rusness, D.G. *Pestic. Biochem. Physiol.* **1986,** *26*, 323-42.
42. Lamoureux, G.L.; Rusness, D.G. *Pestic. Biochem. Physiol.* **1987,** *27,* 318-29.
43. Ezra, G.; Dekker, J.H.; Stephenson, G.R. *Weed Sci.* **1985,** *33*, 287-90.
44. Zorner, P.S.; Sheppard, B.R. *Weed Sci. Soc. Amer. Abstracts* **1985,** *25*, 290.
45. Ray, P.G.; Markley, L.D.; Snelling, J.; Coleman, S. *Weed Sci. Soc. Amer. Abstracts,* **1990,** *30*, 290.
46. Zorner, P.S. pers. comm. 1989.
47. Lamoureux, G.L.; Rusness, D.G. In *Glutathione-Chemical Biochemical and Medical Aspects*, Dolphin, D.; Poulsen, R.; Avromovic, O., Eds.; Coenzymes and Cofactors Wiley: New-York 1989, Vol. 2 part B; pp. 153-96.
48. Ommen, B.v.; Besten, C.d.; Rutten, A.L.M.; Ploemen, J.H.T.M.; Vos, R.M.E.; Müller, F.; Bladeren, P.J.v. *J. Biol. Chem.* **1988,** *263*, 12939-42.
49. Caseley, J.C.; Copping, L.; Mason, D. In *Herbicide Resistance in Weeds and Crops* , Caseley, J.C.; Cussans, G.W.; Atkin, R.K., Eds.; Butterworth: Oxford, 1990, p. 429-30.
50. Wood, E.J.; deVillar, M.I.P.; Melgar, F.J.; Zerba, E.N. *Pestic. Biochem. Physiol.* **1986,** *26*, 170-82.
51. O'Keefe, D.; Romesser, J.A.; Leto, K.J. In *Phytochemical Effects of Environmental Compounds,* Saunders, J.A.; Channing, L.K.; Conn, E.E., Eds.; Plenum: New York, 1987, pp. 151-73.
52. Gressel, J. *Wheat Herbicides - The Challenge of Emerging Resistance,* Biotechnology Affiliates: Checkendon, Reading, U.K., 1988, 247 p.
53. McFadden, J.J.; Frear, D.S.; Mansager, E.R. *Pestic. Biochem. Physiol.* **1989,** *34,* 92-100.
54. Frear, D.S.; Swanson, H.R.; Thalacker, F.W. *Pestic. Biochem. Physiol.* **1991,** *41*, 274-87.
55. Frear, D.S.; Swanson, H.R.; Tanaka, F.S. *ACS Natl. Mtg. 203, Div. Agrochem. Abstract,* No. 113, 1992.
56. Burnet, M.W.M.; Holtum, J.A.M.; Powles, S.B. *Weed Sci. Soc. Amer. Abstracts*, **1992,** *32*, 166.
57. McFadden, J.J.; Gronwald, J.W.; Eberlein, C.V. *Biochem. Biophys. Res. Comm.* **1990,** *168*, 206-13.
58. Moreland, D.E.; Corbin, F.T.; Novitzky, W.P; Parker, C.E.; Tomer, K.B. *Z. Naturforsch.* **1990,** *45c,* 558-64.

59. Moreland, D.E.; Corbin, F.T. *Weed Sci. Soc. Amer. Abstracts* **1990**, *30*, 179.
60. Mougin, C.; Cabanne, F.; Canivenc, M.-C.; Scalla, R. *Plant Sci.* **1990**, *66*, 195-203.
61. Fonné-Pfister, R.; Kreuz, K. *Phytochemistry* **1990,** *29,* 2793-6.
62. Fonné-Pfister, R.; Gaudin, J.; Kreuz, K.; Ramsteiner, K.; Ebert, E. *Pestic. Biochem. Physiol.* **1990**, *37*, 165-73
63. Hamerski, D.; Matern, U. *FEBS Lett.* **1988**, *239*, 263-5.
64. Matern, U.; Wendorff, H.; Hamerski, D.; Pakusch, A.E.; Kneusel, R.E. *Bull. Liason Groupe Polyphenols* **1988**, *14,* 173-84.
65. Cole, D.J.; Owens, W.J. *Plant Sci.* **1987**, *50,* 13-20.
66. Cole, D.J.; Loughman, B.C. *Physiol. Veg.* **1985**, *23,* 879-86.
67. Cabanne, F.; Huby, D.; Gaillardon, P.; Scalla, R. *Pestic. Biochem. Physiol.* **1987**, *28*, 371-80.
68. Gaillardon, P.; Cabanne, F.; Scalla, R.; Durst, F. *Weed Res.* **1985,** *25*, 397-402.
69. Gonneau, M.; Pasquette, B.; Cabanne, F.; Scalla, R.; Durst, F. *Weed Res.* **1988,** *28*, 19-25.
70. Kemp, M.S.; Caseley, J.C. *Proc. Brit. Crop Protect. Conf.- Weeds,* 1987, pp. 895-99.
71. Leah, J.M.; Worrall, T.L.; Cobb, A.H. *Pestic. Biochem. Physiol.* **1991,** *39*, 232-9.
72. Moore, B.A.; Zorner, P.S.; Carlson, D.R., Winkler, V.W. *Weed Sci. Soc. Amer. Abstracts* **1990**, *30,* 59.
73. Neighbors, S.; Privalle, L.S. *Pestic. Biochem. Physiol.* **1990**, *37*, 145-53.
74. Varsano, R.; Rubin, B. *Phytoparasitica*, **1991**, *19*, 225-36.
75. Powles, S.B.; Lilegren, D. *Weed Sci. Soc. Amer. Abstracts*, **1988**, *28,* 187.
76. Kudsk, P.; Thonke, K.E.; Streibig, J.C. In *Adjuvants and Agrochemicals,* Chow, P.N.P.; Grant, C.A.; Hinshalwood, A.M.; Simundsson, E.; Eds., CRC Press: Boca-Raton, 1989, Vol. 1,pp. 175-81.
77. Metcalf, R.L.; McKelvey, J.J. *Insecticide Synergism in the Future for Insecticides. Needs and Prospects,* 1979, Wiley; New York.
78. Blee, E.; Durst, F. *Arch. Biochem.* **1987**, *254,* 43-52.
79. Belai, I.; Matolcsy, G. *Pestic. Sci.* **1988**, *24*, 205-19.
80. Vanden-Bossche, H.; Marichal, P.; Gorrens, J.; Bellens, D.; Verhoeven, H.; Coene, M.C.; Lauwers, W.; Janssen, P.A.J. *Pestic. Sci.* **1987**, *21*, 289-306.
81. Moreland, D.E.; Corbin, F.T. *Weed Sci. Soc, Amer. Abstracts,* **1992**, *32*, 187.
82. Prochaska, H.J.; Talalay, P. *Cancer Res.,* **1988**, *48*, 4776-4782.
83. Rademacher, W.; Fritsch, H.; Graebe, J.E.; Sauter, H.; Jung, J. *Pestic. Sci.* **1987**, *21*, 241-52.
84. Gressel, J. In *Weed Physiology,* Duke, S.O., Ed.; CRC Press: Boca-Raton, 1985, pp. 159-89.
85. Summers, L.A. *The Bipyridilium Herbicides,* Academic Press: New York, 1980, 449 p.
86. Matringe, M.; Camadro, J.M.; Labbe, P.; Scalla, R. *Biochem. J.* **1989**, *260,* 231-5.
87. Witkowski, D.A.; Halling, B.P. *Plant Physiol.* **1989,** *90*, 1239-42.
88. Hartman, P.E.; Suzuki, C.K.; Stack, M.E. *Appl. Environ. Microbiol.* **1989,** *55*, 7-14.
89. Martin, J.P.; Logsdon, N. *J. Biol. Chem.* **1987**, *262,* 7213-9.
90. Ben-Amotz, A.; Gressel, J.; Avron, M. *J. Phycol.* **1987**, *23*, 176-81.
91. Finckh, B.F.; Kunert, K.J. *J. Agric. Food Chem.* **1985,** *33*, 574-7.
92. *Free Radicals in Biology and Medicine,* Halliwell, B.; Gutteridge, J. Eds; Oxford Press: Oxford, 1985, 346p.

93. Asada, K.; Takahashi, M. In *Photoinhibition,* Kyle, D.J.; Osmond, C.B.; Arntzen C.J. Eds.; Elsevier: Amsterdam, 1987, pp. 227-87.
94. Shaaltiel, Y.; Gressel, J. *Plant Physiol.* **1987**, *85,* 869-71.
95. Shaaltiel, Y.; Chua, N.-H.; Gepstein, S.; Gressel, J. *Theor. Appl. Genet.* **1988**,*75,* 850-6.
96. Jansen, M.A.K.; Malan, C.; Shaaltiel, Y.; Gressel, J.; *Z. Naturforsch.* **1990,** *45c*, 463-9.
97. Asada, K.; Nakano, Y.; Hossain, M.A. In *Oxidative Damage and Related Enzymes.* Rotilio, G.; Bannister, J.V. Eds.; Harwood: Chichester, 1984, pp. 342-347.
98. Gressel, J.; Shaaltiel, Y. In *Biotechnology in Crop Protection.* Hedin, P.A.; Menn, J.J.; Hollingworth, R. M., Eds.; Amer. Chem. Soc.: Washington, 1988, pp. 4-24..
99. Bewick, T.A.; Stall, W.M.; Kostewicz, S.R.; Smith, K. *Weed Technol.* **1991**, *5,* 61-5.
100. Warshawsky, A.; Cohen, N.; Gressel, J. Unpublished results, 1992.
101. Gazit, Y.; Ishaaya, I.; Perry, A.S. *Pestic. Biochem. Physiol.* **1989,** *34,* 103-10.
102. Ishaaya, I.; Ascher, K.R.S.; Casida, J.E.; *Crop. Protec.* **1983**, *2***,** 335-43.
103. Krauskopf, B.; Fedtke, C.; Feucht, D. In *Proc. 4th EWRS Mediterranean Symp.* 1989.
104. Klamroth, E.E.; Fedtke, C.; Kühbauch, W.C. *Weed Sci.* **1989**, *37*, 517-20.
105. Fedtke, C.; Strang, R.H. *Z. Naturforsch.* **1990**, *45c* , 565-7.
106. Blumhorst, M.R.; Kapusta, G. *Weed Tech.* **1987**, *1*, 149-52.
107. Gressel, J.; Segel, L.A. *Z. Naturforsch.* **1990**, *45c,* 470-3.
108. Durner, J.; Thiel, A.; Böger, P. *Z. Naturforsch.* **1986**, *42c***,** 881-4.
109. Ludlum, C.T.; Felton, G.W.; Duffey, S.S. *J. Chem. Ecol.* **1991**, *17*, 217-37.
110 Sharon, A.; Ghirlando, R.; Gressel, J. *Plant Physiol.* **1992**, *98,* 303-8.
111. Sharon, A.; Amsellem, Z.; Gressel, J. *Plant Physiol.* **1992**, *98*, 654-9.

RECEIVED July 22, 1992

Chapter 5

The Porphyrin Pathway as a Herbicide Target Site

Ujjana B. Nandihalli[1] and Stephen O. Duke[2]

**[1]Center for Alluvial Plains Studies, Delta State University,
Cleveland, MS 38733
[2]Southern Weed Science Laboratory, Agricultural Research Service,
U.S. Department of Agriculture, P.O. Box 350, Stoneville, MS 38776**

The porphyrin pathway is one of the most important metabolic pathways in plants. Nevertheless, this pathway has been virtually overlooked as a herbicide target site in biorational design strategies. For over a decade, several pyridine and phenanthroline analogues were known to interfere with the porphyrin pathway by inducing porphyrin accumulation in treated plants. The potential of δ-aminolevulinic acid (ALA), a porphyrin precursor, as an environmentally safe herbicide has been studied extensively. Plants treated with ALA either alone or in combination with the chemical modulators accumulate large amounts of different types of porphyrin compounds. Excessive amounts of porphyrins injure plants by type II photoperoxidation. Recently, numerous commercial herbicides such as diphenyl ethers have been found to cause accumulation of protoporphyrin IX by inhibiting protoporphyrinogen oxidase, the last enzyme common to both heme and chlorophyll synthesis. In this article, we review the modes of action of herbicides that interfere with the porphyrin pathway and analyze the potential of the porphyrin pathway for biorational herbicide design.

In the past, the reduction of chlorophyll (Chl) content in herbicide-treated plants has been used as a quantitative measurement of herbicide phytotoxicity. The reduced Chl levels were thought mostly to be due to "secondary effects." The porphyrin pathway as a direct herbicide target site has been of strong interest only during the last 8 years due to two major findings. First, δ-aminolevulinic acid (ALA) was found to act as potent herbicide in combination with synthetic modulators of the porphyrin pathway (*1*). Second, several major classes of herbicides were found to act through inhibition of protoporphyrinogen oxidase (Protox), resulting in the accumulation of large amounts of photodynamic protoporphyrin IX (Proto IX) (*2-4*). The widespread use of herbicides for controlling weeds has raised serious environmental concerns. The porphyrin pathway is much more active in plants than in animals because of the requirement for continued Chl synthesis. Furthermore, those parts of the pathway exclusively committed to Chl synthesis are not found on animals. ALA is a natural compound

0097–6156/93/0524–0062$06.00/0

present in both palnts and animals. ALA and its products (porphyrins) degrade rapidly in the environment, thus causing no deleterious effects on ecosystems. Thus, the porphyrin pathway is a potential site for biorational design of toxicologically benign herbicides. In this review, we discuss ALA-based as well as Protox-inhibiting synthetic herbicides and the potential of other enzymes in the porphyrin pathway as herbicide target sites.

The Porphyrin Pathway

The enzyme properties and catalytic reactions of the porphyrin pathway have been reviewed recently (*5*) (Figure 1). ALA is the first committed precursor of the porphyrin pathway which provides all the carbon and nitrogen required for porphyrin synthesis. The synthesis of ALA is also considered to be the rate-limiting step in the biosynthesis of Chl. Porphobilinogen (PBG) is formed by the condensation of two ALA molecules by ALA dehydratase. Uroporphyrinogen III, coproporphyrinogen III, and protoporphyrinogen IX (Protogen) are "porphyrinogens" which are non-fluorescent and non-photosensitizing compounds. Their macrocycles are non-aromatic and non-planar. The compounds from Proto IX to monovinyl-protochlorophyllide (MV Pchlide) are called porphyrins. These are fluorescent and photosensitizing structures, containing aromatic and planar macrocycles. The formation of chlorophyllide (Chlide) from either MV or divinyl (DV) Pchlide requires light; therefore, the porphyrin pathway halts at the Pchlide level in darkness. The biochemical aspects of the enzymes of the porphyrin pathway are poorly understood. Most of the steps in the porphyrin pathway are catalyzed by multiple enzyme systems.

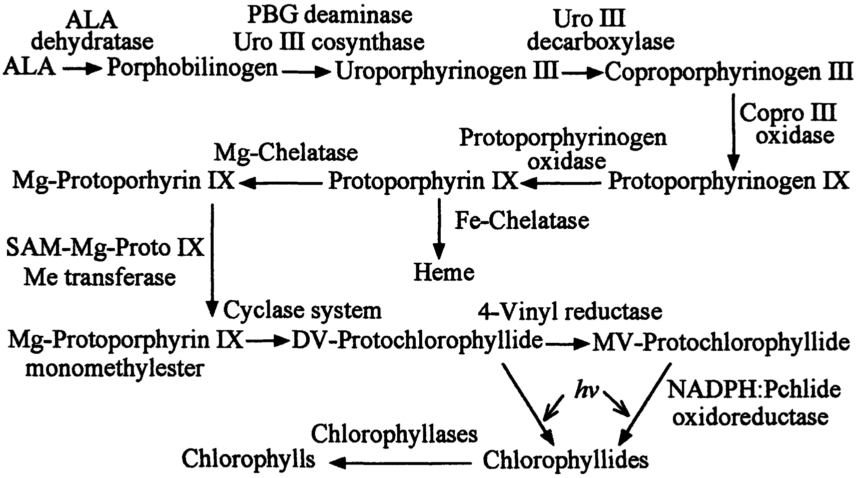

ALA dehydratase
PBG deaminase
Uro III cosynthase
Uro III decarboxylase
ALA
Porphobilinogen
Uroporphyrinogen III
Coproporphyrinogen III
Copro III oxidase
Mg-Chelatase
Protoporphyrinogen oxidase
Mg-Protoporhyrin IX
Protoporphyrin IX
Protoporphyrinogen IX
Fe-Chelatase
SAM-Mg-Proto IX Me transferase
Heme
Cyclase system
4-Vinyl reductase
Mg-Protoporphyrin IX monomethylester
DV-Protochlorophyllide
MV-Protochlorophyllide
hv
NADPH:Pchlide oxidoreductase
Chlorophyllases
Chlorophylls
Chlorophyllides

Figure 1. The porphyrin biosynthetic pathway in higher plants (*5*).

Porphyric Herbicides

To be classified as a porphyric herbicide (PH), the chemical must be able to cause the accumulation of large amounts of one or more porphyrin compounds in treated plants either in darkness or in the light. The porphyrins are photosensitizers and excessive levels of porphyrins in plant cells lead to the production of reactive singlet oxygen ($^1O_2$) under light. $^1O_2$ reacts with the membrane lipids by a type II photodynamic process, resulting in membrane lipid peroxidation, membrane breakdown and ultimately, tissue death. Several reviews have appeared recently on both the natural ALA-based PH (*6-8*) and synthetic PHs that exclusively inhibit plant Protox (*9-12*).

ALA as a Natural Porphyric Herbicide

In untreated plants kept in darkness, only a small amount of Pchlide accumulates due to the limited availability of ALA in darkness. When these plants are irradiated, Pchlide is rapidly and almost fully photoconverted to Chlide which is then utilized in the synthesis of Chl, causing no photodynamic injury to the plant. Long before the introduction of the PH concept by Rebeiz *et al.* (*1*), it was known that plant leaves incubated with exogenous ALA accumulated large amounts of Pchlide in the dark (*13*). These leaves were bleached when irradiated with white light. Furthermore, Proto IX and Mg-Proto IX Me were accumulated by barley leaves that were simultaneously treated with α,α'-dipyridyl (DPY) and ALA. Duggan and Gassman (*14*) found that several iron-complexing compounds induce porphyrin buildup in treated plant tissue. These compounds were collectively termed "modulators" by Rebeiz et al. (*1*).

Porphyrin Photodegradation and Herbicidal Effects. The porphyrins that accumulate in the dark in response to ALA or ALA+modulator treatments are not rapidly utilized in the formation of Chl when plants are exposed to the light. It is believed that a large proportion of the loss of accumulated porphyrins during exposure to light in these treatments is due to photodegradation rather than movement to Chl. In ALA+DPY-treated cucumber plants kept in darkness for 17 h, both Pchlide and Mg-Proto IX equivalents (MPE) were degraded differently when irradiated (*1*). Sixty-five percent of the dark-accumulated Pchlide was lost within 30 min of irradiation and it took 4 h to reach the levels found in untreated plants. The dark-accumulated MPE level decreased rapidly within 30 min of irradiation, followed by an increase for up to 2 h and then decreased to undetectable levels after 4 h. Recently, Chakraborty and Tripathy (*15*) demonstrated that the chloroplasts isolated from plants treated with ALA and kept in darkness for 14 h lost 40% of dark-accumulated Pchlide within 15 min of irradiation and further exposure for up to 1 h did not affect the Pchlide concentration. These high levels of dark-accumulated non-phototransferable porphyrins in plants are responsible for the peroxidation of the membrane lipids. Herbicidal injury occurs rapidly, usually being detectable within the first 20 min of irradiation of dark-incubated plants. Initially, isolated water soaked spots are seen on tissue, followed by loss of leaf turgidity and desiccation.

Herbicidal Synergism Between ALA and Modulators. In the ALA alone treatment, Pchlide is the only porphyrin that accumulates, but in ALA + modulator treatments several other types of porphyrins are accumulated, depending upon the target site of the modulator. The total porphyrin content present in plant tissue before exposure to light and the eventual tissue damage in the light are correlated (Table I). Also, the modulators typically interact with ALA in a synergistic fashion with respect to both porphyrin accumulation and herbicidal damage.

Table I. Synergistic interaction of ALA (5 mM) and picolinic acid (20 mM) on porphyrin production and herbicidal damage in cucumber. Five-day-old seedlings were sprayed with the chemicals and incubated in darkness for 20 h before determination of porphyrins and exposure to light. Herbicidal injury was evaluated 24 h after exposure to the light (Nandihalli and Rebeiz, unpublished data)

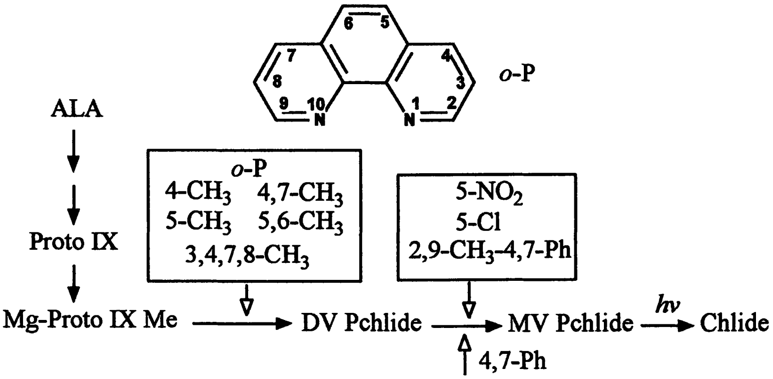
Treatment	Mg-Proto IX Me	Pchlide	Total porphyrins	Herbicide damage
	–(nmoles/100 mg protein)–			(%)
Control	0.7	53	54	0
ALA	2.6	380	383	39
Picolinic acid	8.0	76	84	0
ALA + Picolinic acid	78.5	507	586	95

Classification of Modulators. Based on the types of porphyrins that accumulate in response to modulator treatment, modulators have been classified into four groups (*6*): (a) inducers of porphyrins, which cause porphyrin accumulation in treated plants *alone*, (b) enhancers of conversion of exogenous ALA into MV Pchlide, (c) enhancers of conversion of exogenous ALA into DV Pchlide, and (d) inhibitors of MV Pchlide accumulation. In the case of (b) and (c), porphyrin contents of ALA+Modulator treatments are significantly greater than that of ALA alone treatment.

Mechanism of Action of Inducer-Type Modulators. Even though chelating agents such as 1,10-phenanthroline (*o*-P) and DPY have been utilized in the investigations of control mechanisms of Chl biosynthesis since the late 1950s, their biochemical mode of action in plants is not completely understood. The most obvious mechanism is that the chelators inhibit heme synthesis by chelating free Fe^{++}, thus diverting Proto IX solely into the magnesium porphyrin pathway. However, Duggan and Gassman (*14*) found this reasoning to be invalid because heme levels in the untreated bean leaves were not high enough (<1% of porphyrins accumulated in treated tissue) to account for the increased porphyrin levels in the chelator-treated tissue. Even though it has not been suggested as a possible mode of action of chelators, it is possible that the inhibited heme synthesis might stimulate ALA formation since heme is known to be a feed-back inhibitor of ALA synthesis (*5*). The level of ALA synthetase escalates when heme levels drop in the cell (5). Duggan and Gassman (*14*) proposed two possible mechanisms for chelator action; (a) chelators stimulate ALA synthesis in the dark by inhibiting iron-protein enzymes that inhibit the activity of ALA-synthesizing enzymes, and (b) iron may be necessary for the enzyme which converts Mg-Proto IX Me to Pchlide, and the chelators render iron unavailable for the enzyme of this reaction. This was later confirmed by experiments in which the inhibitory effects of chelators on this step were reversed by either administering tissue with metal salts (Fe, Zn, and Co) or washing tissue in iron-containing buffers (*16, 17*).

The above hypotheses were centered around the chelating behavior of modulators. However, the non-chelating isomer of *o*-P, *m*-P, and its analogue, phenanthridine were as active as *o*-P in inducing porphyrin accumulation (*18*), suggesting that the chelating property alone may not be responsible for their porphyrin-induction phenomenon.

In a dark-porphyrin accumulation study (*19*) involving analogues of *o*-P, it was found that the analogues inhibited or stimulated different steps in the porphyrin pathway as depicted below:

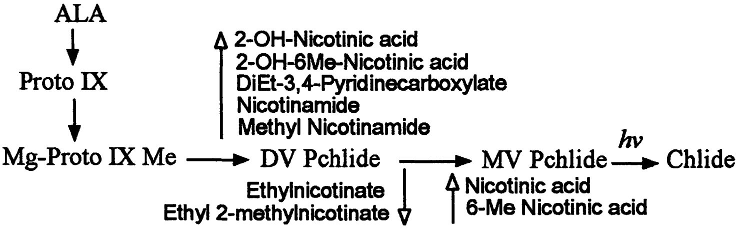


The substitutions on the *o*-P molecule with either one, two, or four methyl groups acted at the same site as *o*-P, but the structures with a nitro, chloro, or two methyl plus two phenyl groups acted at a different site than the *o*-P. Furthermore, the substitution of *o*-P with two phenyl rings induced the accumulation of mainly MV-Pchlide, indicating that the 4,7-Phenyl (4,7-Ph) substitution either stimulated the enzyme which converts DV-Pchlide to MV-Pchlide or prevented degradation of MV Pchlide. These results support the conclusion of Bednarik and Hoober (*18*) that the chelation property may not be the only factor involved in the interaction of chelating compounds such as *o*-P with the porphyrin pathway.

Based on the screening and biochemical research on porphyrin biosynthesis modulators, it has been suggested that the modulators may interact directly with the enzymes of the porphyrin pathway (*7*, *8*, *19-21*) by binding to or close to the receptor site. The compounds that interfered with the porphyrin pathway invariably fell into two separate categories: (a) those that were structurally related to one half fraction of a tetrapyrrole molecule such as diphenyl ethers, phenopylates, dipyridyls, and phenanthrolines, and (b) those that were structurally related to one quadrant of a tetrapyrrole molecule such as picolinic acid, nicotinic acid, and substituted pyridyls.

Additional effects of the induction-type modulators on biochemical and physiological processes have been reported. When pea plants were treated with *o*-P for 14 h in darkness and then exposed to the light, net photosynthesis was reduced by 92% (*22*, *23*). *o*-P can also bind to Mg^{++}, and immediately after exposure to the light there may be an insufficient amount of Mg^{++} for carboxylase activation and, thus, photosynthesis is inhibited. The bidentate chelators such *o*-P and DPY inhibited ALA synthesis in corn leaves in the light by 50 to 75%, whereas inhibition in the dark was insignificant (*24*). This was in disagreement with the results of Duggan and Gassman (*14*) who found the chelators to stimulate ALA synthesis in bean leaves. Thus, the chelator effects appear to be species-dependent. The bidentate chelators reduce PBG catabolism in corn, thus increasing the availability of PBG for the synthesis of porphyrins (*24*).

Mechanism of Action of Enhancer-Type Modulators. An effort to understand the action mechanism of modulators which stimulate exogenous ALA incorporation into porphyrins is lacking. The effects of various analogues belonging to the nicotinic acid and nicotinamide groups on the porphyrin pathway have been examined (Nandihalli and Rebeiz, unpublished), especially their role in the enhancement of incorporation of exogenous ALA into porphyrins. Five-day-old cucumber seedlings were sprayed with 5 mM ALA and 10 to 30 mM concentrations of modulators. Plants were incubated in darkness for 20 h before the determination of porphyrin contents and exposure to light for phytotoxicity development. Based on the types of porphyrins they caused to accumulate, their potential target sites were assigned as shown below. The arrows next to the modulators pointing upward indicate a stimulatory effect and that pointing downward indicates an inhibitory effect at that site.



None of the analogues belonging to either groups induced porphyrin accumulation when applied alone at up to 30 mM. The Pchlides were the only porphyrins to accumulate. Even though it is difficult to assign the exact biochemical role(s) for these modulators in the enhanced ALA utilization, it is possible that they stimulate enzyme activity by acting as cofactor analogues. For example, these enzymes require NADPH which contains a nicotinamide moiety in its structure. Furthermore, nicotinate is one of the substrates for the synthesis of NAD, which is a substrate for NADP.

Species Selectivity to ALA Treatment. Since ALA-based herbicide combinations acted via the Chl biosynthetic pathway and since the latter was such a fundamental process common to all plants, it was thought that ALA-based herbicides would not be species selective (*1*). Although, monocots such as corn, wheat, oat, and barley accumulated significant amounts of porphyrins in response to ALA-based herbicide treatment, they suffered minimal herbicidal damage (*1*). In contrast, dicots such as cucumber, lambsquarter, redroot pigweed, and common purslane were severely damged by the same treatment. Species selectivity was attributed to the existence of a multibranched Chl pathway (*25*) and the various species-specific greening groups (*6*). Averina *et al.* (*26*) also found that monocots were less injured by the ALA+DPY spray than dicots, but contrary to Rebeiz *et al.* (*1*), they found that treated monocot plants accumulated significantly less amounts of porphyrins than dicot plants. There were also intra-species differences in susceptibility to applied treatments (*1*, *26*). Different organs of plants such as leaves, stems, and cotyledons accumulated varying levels of porphyrins, possibly due to unequal permeability of these organs to the herbicide. It was suggested that tolerance to ALA treatment could be achieved by breeding crops for high superoxide dismutase activity since superoxide is known to be involved in ALA-induced toxicity (*27*). However, the evidence for the predominant involvement of $^1O_2$ as the primary toxic oxygen species in ALA-induced toxicity has been clearly demonstrated recently (*15*).

Synergism Between ALA and Protoporphyrinogen Oxidase-Inhibiting Herbicides. One of the most attractive propositions for reducing environmental effects of synthetic herbicides is to include a synergizing natural chemical such as ALA in the weed control program that contains Protox-inhibiting herbicides. By doing this, the greater potency of synthetic herbicides are exploited; at the same time their application rates are minimized. Studies have shown a synergistic interaction between ALA and diphenyl ether herbicides such acifluorfen (AF) with respect to Proto IX production. In cucumber plants, ALA plus AF-Na treatment caused substantially greater accumulation of Proto IX than the treatments in which each chemical was applied separately (*28*). Similar results were obtained by Lydon and Duke (*2*) in excised cucumber leaf discs incubated with ALA plus AFM. However, research examining the synergistic herbicidal effects between ALA and Protox-inhibiting herbicides at the whole plant level has not been conducted extensively.

Current Limitations for the Use of ALA as a Herbicide. Four factors limit the development of ALA as a herbicide. First, suitability of a chemical for commercial application needs to be evaluated by conducting multiyear-multilocation field trials. The present high cost of ALA deters conducting not only field efficacy trials but also extensive greenhouse screening studies. However, small-scale field trials conducted to evaluate broadleaf weed control in bluegrass lawn and the defoliation of apple trees (*6*) have shown promise for ALA-based herbicides. Second, the ALA-based herbicides must include a chemical modulator, and the presently known effective modulators may not be environmentally safe. Even though nicotinic acid analogues were not as effective as chelator-type modulators as ALA synergists, these structures could be used as lead compounds in the design of more effective and safer modulators. Third, the requirement of a long post-spray dark-period for ALA-based herbicides forces application to be made at a certain time of the day. This is not practical under large scale farming systems where time is a critical factor. However, the efficacy of these herbicides applied directly in the light without a post-spray dark period has not been tested sufficiently. Fourth, a suitable formulation for maximal cuticular absorption of ALA-based herbicides is not available, which creates difficulty in assessing the true efficacy of these herbicides at the whole-plant level.

Protoporphyrinogen Oxidase as a Herbicide Target Site

Even though several photobleaching herbicides such as diphenyl ethers (DPE) have been in commercial use since the early 1970s, the location of their target site in the porphyrin pathway was discovered recently (*2-4*). The DPE and certain other classes of herbicides cause rapid peroxidative bleaching and/or desiccation of plant tissues (*3, 29-42*). The primary site of action of these herbicides is protoporphyrinogen oxidase (Protox) (*37, 40-42*), the last common enzyme in the synthesis of both heme and chlorophylls (Figure 1). A question arises is how the inhibition of Protox leads to the accumulation of the product of the enzyme, Proto IX, and why the Proto IX is not converted to later intermediates. A detailed mechanism explaining the accumulation of Proto IX in the presence of photobleaching herbicides has been proposed recently by Jacobs et al. (*43*) (Figure 2). By inhibiting Protox, these herbicides induce Protogen accumulation which diffuses out of the plastid envelope into the cytoplasm. Although the Protogen may spontaneously oxidize to Proto IX, there is evidence that the Protogen is converted rapidly to Proto IX by a Protogen-oxidizing factor located at extraplastidic sites such as the plasma membrane. This factor may or may not be enzymic (*43*). By the action of this factor, high levels of Proto IX build up in extraplastidic sites (*44*); some of this may re-enter the porphyrin pathway but most of it accumulates and appears to participate in type II photoperoxidation process which damages plants.

Protox is a membrane-bound enzyme associated mainly with the plastid envelope (*45*). The enzyme is found in both plastidic and mitochondrial fractions

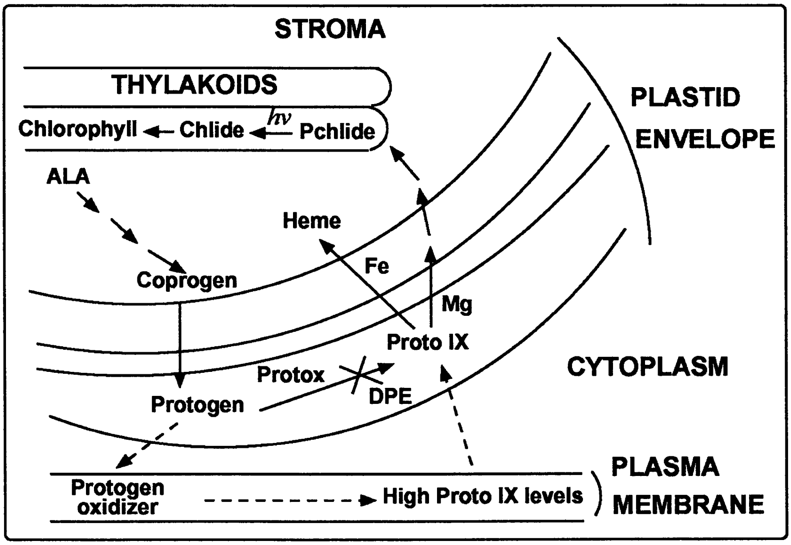


Figure 2. Proposed mode of action of diphenyl ether herbicides. Reproduced with permission from reference 43.

of etiolated plants (*46*). The enzyme from both the organelles appears to be identical in kinetic and physical properties (*46*). Furthermore, Protox from mustard, cucumber, and morningglory was equally sensitive to AF *in vitro*, suggesting that differential enzyme susceptibility may not be involved in the selectivity of peroxidizing herbicides (*47*). However, differential susceptibility of these species to AF is due to differences in Proto IX accumulation, perhaps because some species are able to degrade Proto IX or Protogen faster than others.

Herbicide-induced Proto IX accumulation is very rapid, being easily detected within 15 to 30 min after exposure of plant tissue to the herbicide (*48, 49*). In intact plants treated with AF, significantly greater amounts of Proto IX accumulate in the light than in the dark (*28, 50, 51*). Higher levels of Pchlide and heme (both feed-back inhibitors of ALA synthesis) in darkness limit the effect of Protox inhibitors in darkness (*51*).

Correlations Among Protox Inhibition, Proto IX Accumulation, and Herbicidal Activity. With several peroxidizing herbicides, strong correlations have been observed between Proto IX accumulation and subsequent herbicidal damage in intact as well as excised tissue experiments (*37, 39, 48, 50, 52*). We found very good correlations between Protox-inhibitory (I_{50}), Proto IX induction, and herbicidal activities of 14 phenopylate analogues in barley (*39*). On the contrary, very poor correlations were found between the same biological parameters in a DPE series of compounds, suggesting differential movement of herbicides and/or the alteration of chemistry during the course of uptake and movement of molecules to the active sites in plant tissues (*20*).

Competitive inhibition. The herbicidal Protox inhibitors apparently inhibit the Protox enzyme by competitive inhibition (*53*). From enzyme kinetics studies, AF and three analogues were found to competitively inhibit, with respect to Protogen, the Protox activity from mouse liver mitochondria, corn mitochondria, and corn

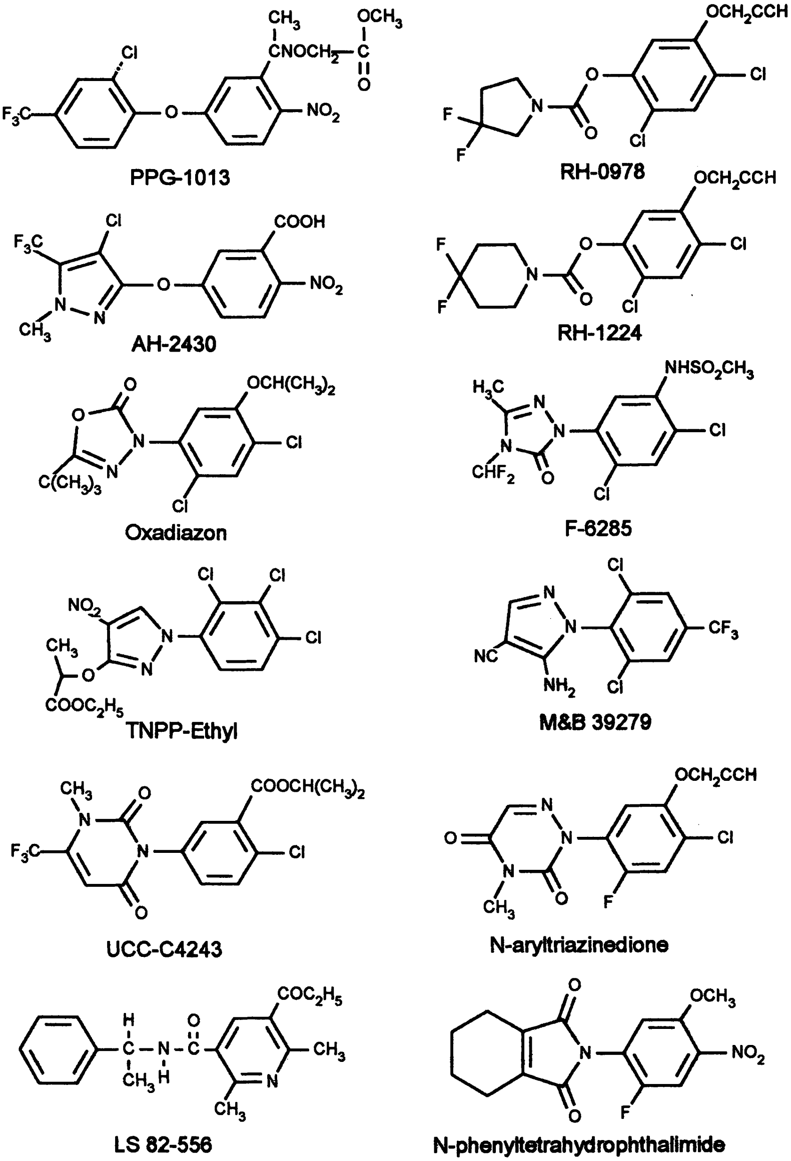
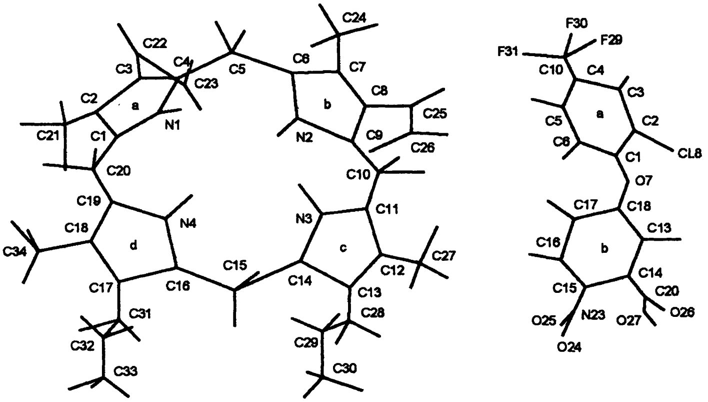

PPG-1013
RH-0978
AH-2430
RH-1224
Oxadiazon
F-6285
TNPP-Ethyl
M&B 39279
UCC-C4243
N-aryltriazinedione
LS 82-556
N-phenyltetrahydrophthalimide

Figure 3. Protox-inhibiting herbicides from different chemical classes.

etioplast membranes. In binding studies, both Protogen and Protox-inhibiting herbicides displaced bound ^{3}H-AF from corn etioplast preparations (*54*) Similarly, five phenopylate Protox-inhibiting analogues competed with ^{14}C-AF for binding sites on Protox (*21*). The specific binding constants and Protox inhibitory activities (I_{50}) of these analogues were highly correlated.

Molecular Similarities Between the Protogen and Protox Inhibitors. For an inhibitor to compete with the substrate for the same active site on the enzyme it must have structural similarity with some part of the substrate molecule. From enzyme kinetic and radiolabeled herbicide binding experiments it was apparent that the Protox inhibitors were competitive inhibitors of Protox. In an attempt to draw molecular similarities between the Protogen and Protox-inhibiting herbicides, we proposed that the Protox inhibitors must be bicyclic, representing approximately one half fraction of the Protogen molecule for proper geometrical fit into the active site. This hypothesis was developed from the observations that (a) all the known Protox inhibiting herbicides belonging to different chemical classes are bicyclic (Figure 3), and (b) the monocyclic compounds such as picolinic acid and pyrrole-2-carboxaldehyde which are found to interfere with the porphyrin pathway did not inhibit Protox.

The hypothesis was tested by comparing the semiempirical molecular properties of Protogen and several analogues of diphenyl ether and phenopylate herbicide classes. It was found that the three dimensional (lengths of x, y, and z coordinates) geometry and van der Waals volume (bulk parameter) of the most active compounds from both herbicide groups matched more closely with the one half structure of the Protogen molecule than the least active analogues. Detailed structural comparisons between Protogen and AF revealed that both the bond and torsion angles of AF at the ether oxygen matched closely with the angles at the methylene bridge between rings B and C of Protogen structure (Figure 4)(*20*). The nucleophilic superdelocalisabilty of AF and Protogen were the same, while the molecular electronic and nuclear energies of AF were one half the levels of Protogen. These results supported the hypothesis that the bicyclic Protox-inhibiting herbicides compete with the Protogen molecule by matching its one half structure (Figure 4).



PROTOPORPHYRINOGEN IX ACIFLUORFEN

Figure 4. Three dimensional structures of Protogen and acifluorfen optimized by a quantum mechanics method (*20*).

Quantitative Structure-Activity Relationships (QSAR)

Diphenyl Ether Analogues. In a QSAR analysis of 24 DPE herbicides, the electronic properties (partial charge, superdelocalisability of the lowest unoccupied molecular orbital, and dipole moment) and lipophilicity (log *P*) properties best accounted for the variation in the Protox-inhibiting activity (I_{50}) (*20*). The Protox I_{50} values ranged from 0.008 to 420 μM. The molecular bulk and overall electrostatic potentials were most responsible for the observed variation in the excised tissue herbicidal effects. In general, the analogues with substitutions on the 3'-position (*m*-carbon) of the *p*-nitrophenyl ring were highly active as both enzyme-inhibitors and herbicides. The activity increased with an increase in surface area and electrostatic potentials of the substituent group. It appears that the active site might display considerable flexibility to the length of this substituent. Two enantiomers with a chiral carbon in the group attached to the 3'-position showed differential activity (100-fold). This differential potency of the chiral compounds further supports the observation that the substituent groups at the 3'-position are important for DPE activity. Neither the enzyme-inhibitory activity nor the initial herbicidal activity were changed by substituting a *p*-chloro for a *p*-nitro group. In the acifluorfen-methyl structure, the herbicidal activity was completely lost when the CF_3 group was moved from the *para* to the *meta* position. Compounds with either sulfide, sulfoxide, or sulfone bridges between the phenyl rings were herbicidally inactive.

Phenopylate analogues. The molecular properties of phenopylate (2,4-dichlorophenyl-1-pyrrolidinecarboxylate) and 13 of its O-phenyl pyrrolidino- and piperidino-carbamate analogues were correlated with their capacity to inhibit Protox, to cause accumulation of Proto IX, and to cause herbicidal injury (*21*). All three biological properties correlated well with the van der Waals volume, electrophilic superdelocalisability, and energy of the lowest unoccupied molecular orbital. The relationships between biological activities and log *P* were non-linear.

N-Phenyltetrahydrophthalimides. Lyga *et al.* (*33*) examined the QSAR of N-phenyl-3,4,5,6-tetrahydrophthalimide analogues using tissue growth inhibition as determined by dry weight reduction (pI_{50}) and greenhouse postemergence visual injury (ED_{50}) data as biological parameters. The molecular properties such as Hansch hydrophobicity constants, parainductive and resonance parameters, and molar refractivity effectively predicted the pI_{50} and ED_{50}. For optimal activity, the *para* position of the phenyl ring (same as *para*-nitro position in DPE) required a small, hydrophobic, electronegative group. Similar substitutional requirement was prevalent in aryltriazinedione Protox-inhibiting herbicides (*34*).

From QSAR analyses of various classes of peroxidizing herbicides, it appears that Protox activity responds primarily to the substitutional modifications on the phenyl ring (*p*-nitrophenyl in DPEs). Furthermore, a substitution at the *meta* position on the *p*-chloro (phenopylate) or *p*-nitro (DPE) ring is essential for greater herbicidal activity. A similar observation was made by Hayashi (*55*) from a QSAR analysis of 22 AKH-7088 (DPE) analogues.

Other Enzymes of the Porphyrin Pathway as Potential Herbicide Target Sites.

Apart from the well-studied porphyrin enzymes that are discussed so far in this review, several other enzymes have been shown to be affected by either some commercial herbicides or by well known, commonly used enzyme inhibitors whose herbicidal properties have not been tested fully. Some of these enzymes and their inhibitors are presented in this section.

ALA Dehydratase. ALA dehydratase catalyzes the formation of PBG from two molecules of ALA (*56*). The enzyme appears to be an attractive herbicide target site because when this enzyme is inhibited the flow of carbon and nitrogen sources essential for Chl synthesis is cut off. Thus, the treated plants are unable to replenish Chl and sustain further growth. Several inhibitors (Figure 5) have been utilized as ALA dehydratase inhibitors in studies that involved regulation of the ALA biosynthesis pathway (*57*). These compounds inhibit the enzyme by competitive inhibition by having a succinyl moiety similar to that found in ALA (indicated within the dashed line in Figure 5).

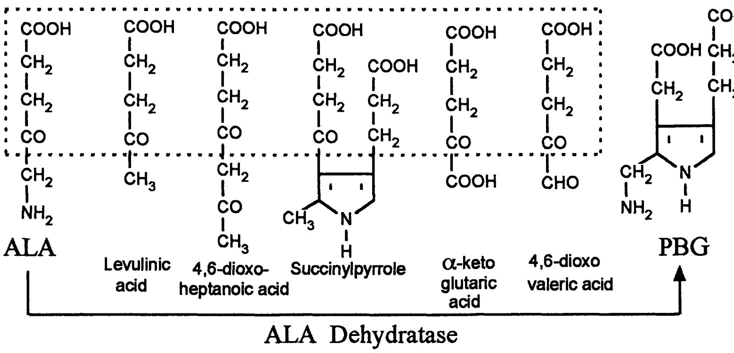


Figure 5. Inhibitors of ALA dehydratase.

PBG Deaminase. PBG deaminase condenses four PBG molecules to form the first tetrapyrrole, hydroxymethylbilane, which is then converted to uroporphyrinogen III by another enzyme, Uro III cosynthase (Figure 1). In barnyardgrass, the herbicide benthiocarb inhibited PBG deaminase activity, resulting in reduced levels of all porphyrins (*58*). Since the enzyme carries thiol groups, the authors speculated that benthiocarb may inhibit the enzyme by interacting with free -SH groups at the enzyme active site.

4-vinyl Reductase. The enzyme catalyzes the reduction of DV Pchlide to MV Pchlide (Figure 1). Nicotinamide inhibits this step in *Rhodobacter spheroides* (*59*) and in the aerobic photosynthetic bacterium *Erythrobacter sp.*, resulting in the accumulation of DV Pchlide. Among 12 nicotinamide derivatives and isomers, only nicotinamide was effective, suggesting that in addition to the completeness of the pyridine ring skeleton at positions 1 to 3, the carboxylic acid amide group is essential for this inhibition. The authors suggested that nicotinamide may act as a competitive inhibitor of this enzyme. A possibility might be that the presence of excess nicotinamide increased the synthesis of NAD so that the increased ratio of NAD/NADH inhibited the enzymatic reduction of 4-vinyl Pchlide to the next step (*60*). In cucumber plants such an inhibition by nicotinamide was absent (Nandihalli and Rebeiz, unpublished).

Does the Substrate Macrocycle Determine the Inhibitor Structure?

By comparing the structures of compounds that inhibit a single enzyme (i.e. Protox) with those compounds that interfere with the enzymes of porphyrin synthesis, there seems to be good agreements between the nature of the substrate macrocycles and the basic backbone structures of inhibitors, as shown in Figure 6.

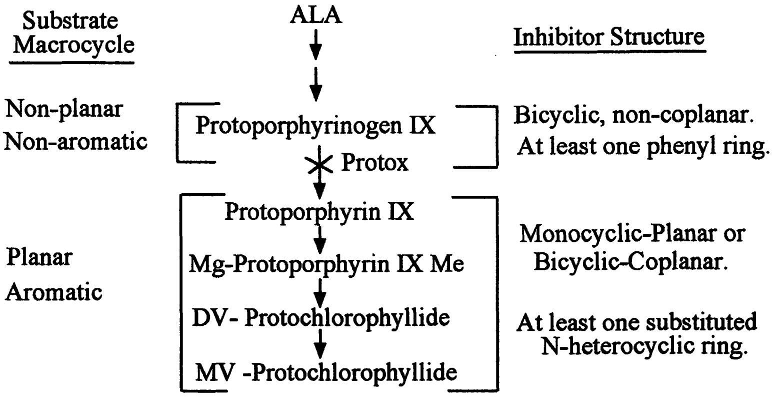

Substrate Macrocycle
ALA
Inhibitor Structure
Non-planar
Non-aromatic
Protoporphyrinogen IX
Protox
Bicyclic, non-coplanar.
At least one phenyl ring.
Protoporphyrin IX
Mg-Protoporphyrin IX Me
DV- Protochlorophyllide
MV -Protochlorophyllide
Planar
Aromatic
Monocyclic-Planar or
Bicyclic-Coplanar.
At least one substituted
N-heterocyclic ring.

Figure 6. Proposed relationships between structures of porphyrin substrate macrocycles and backbone structures of inhibitors.

The Protogen macrocycle is non-planar (therefore two adjacent pyrrole rings are non-coplanar). It appears that because of this non-planarity, the active site on Protox may accommodate only two adjacent pyrrole rings of the Protogen molecule at a time. This hypothesis is supported by the existence of one (tetrahydroprotoporphyrin IX, P-503) (*61*) or most probably two reaction intermediates in the enzymic oxidation of Protogen to Proto IX (Figure 7).

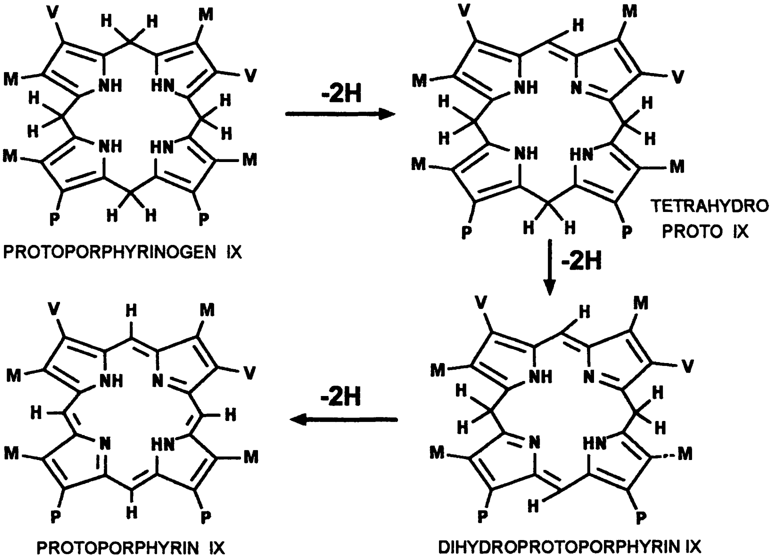

PROTOPORPHYRINOGEN IX
-2H
TETRAHYDRO PROTO IX
-2H
-2H
PROTOPORPHYRIN IX
DIHYDROPROTOPORPHYRIN IX

Figure 7. Proposed reaction pathway of oxidation of Protogen to Proto IX.

In other words, the enzymic reaction appears to be a stepwise process in which various sections of the Protogen molecule are oxidized at different times either by the same or different enzyme molecule(s). All the known Protox-inhibiting herbicides (Figure 3) are bicyclic and the enzyme kinetics and radiolabeled herbicide binding studies of some of these chemical classes have shown that these herbicides reduce Protox activity by competitive inhibition. These herbicides contain non-coplanar rings and therefore they are able to bind to the same site on the Protox as Protogen.

Similar relationships exist between the porphyrins which contain a planar macrocycle and the compounds that regulate porphyrin biosynthesis. All the known compounds that interfere with the synthesis of porphyrins from Proto IX to MV-Pchlide (Figure 6) are either monocyclic and planar (pyridine derivatives) or bicyclic or tricyclic and coplanar (DPY and phenanthroline derivatives). Furthermore, at least one ring is a N-heterocycle. None of these inhibitors affect Protox, further substantiating the requirement of a bicyclic, non-coplanar structure for Protox inhibition.

Prospects for Biorational Design

Millions of compounds have been screened for herbicidal activity in traditional herbicide discovery efforts. Yet, only one enzyme site in this pathway, Protox, has been found to be inhibited by herbicides discovered by this strategy. Many different Protox inhibitors, representing a relatively large number of different chemical classes, have been discovered by screening synthesized compounds for herbicidal activity. Some of these are described above. The Protox inhibitors that have been discovered through traditional discovery programs are generally three orders of magnitude more potent as herbicides than the compounds that have been found to inhibit the earlier or later steps of porphyrin synthesis. The question of why Protox is such a good site for herbicidal action arises.

We think that this could be due to either or both of two factors. First, as mentioned above, there are a relatively large number of compounds that are good competitive inhibitors of Protox. This is probably due to the flexible macrocycle of Protogen versus the rigid macrocycle of all compounds from Proto IX forward in the Chl pathway. The requirement for a coplanar, bicyclic compound greatly restricts the number of possible inhibitors for enzymes utilizing porphyrins, rather than porphyrinogens, as substrates. Nevertheless, one might expect to have found at least one highly potent inhibitor of one of the enzymes with porphyrin substrates. Furthermore, it is curious that no herbicides that inhibit coproporphyrinogen III oxidase or uroporphyrinogen III decarboxylases have been discovered. The answer to these two apparent anomalies may be the same. These enzymes are all thought to be localized within the plastid. Thus, the opportunity for accumulated porphyrins to leave the plastid to occupy less protected parts of the cell (Figure 2) may be minimal compared to the situation with Protox, a primarily plastid envelope-localized enzyme. Why is Protox localized in the plastid envelope? Recent unpublished data of Alison Smith (Cambridge Univ., U.K.) and earlier findings (*46, 62*) suggest that the plant mitochondria contain only the porphyrin synthesis enzymes for converting Protogen to heme. This suggests that the mitochondria, and perhaps, the cytoplasm rely on the plastid for Protogen in order to make Proto IX for heme, phytochrome chromophores, etc. Therefore, the plastid may be primed to export Protogen when Protox is inhibited. Another aspect of accumulation of porphyrins within the plastid versus outside the plastid is that accumulation of porphyrins within the plastid will eventually result in a high concentration of the inhibited enzyme's substrate. With a reversible inhibitor, substrate accumulation will reduce the effectiveness of enzyme inhibition, resulting in the need for higher concentrations of the inhibitor to compete with the substrate. In the case of Protox, the accumulated substrate of the inhibited enzyme is apparently rapidly dissipated, so that a relatively low inhibitor concentration remains effective.

Does this suggest that other sites of the porphyrin pathway are not good herbicide target sites? No, it simply means that the symptomology caused by inhibition of other target sites may develop much more slowly than that caused by Protox inhibitors because accumulation of porphyrins in the chloroplast, which is biochemically equipped to detoxify toxic oxygen species, may not cause the rapid cellular deterioration associated with Protox inhibitors. Structure-activity studies of inhibitors of porphyrin substrate-requiring enzymes could result in the identification of highly effective herbicides that would be specific for organisms dependent on the Chl synthesis. Although such compounds might be slower and require higher application rates, they might offer toxicological advantages due to specificity for enzymes unique to Chl synthesis.

Summary

The porphyrin pathway provides effective sites for herbicide action because of the existence of several modes through which herbicides can interact with the pathway; (a) inhibition of a specific step which prevents the synthesis of porphyrin precursors such as ALA, (b) inhibition of a catalytic step which results in the accumulation of either one or more photosensitizing porphyrins or an immediate porphyrin precursor such as Protogen (which is then converted to Proto IX), (c) stimulation of the biosynthesis of porphyrin precursors such as ALA, and (d) incorporation of exogenously supplied precursors such as ALA into porphyrins. The use of ALA as an environmentally safe herbicide is a very attractive proposition whose suitability as a commercial herbicide by itself or as a synergist of Protox-inhibiting herbicides should be explored. Biorational design of inhibitors of porphyrin substrate-requiring enzymes could result in herbicides that are highly specific for Chl-synthesizing organisms.

Literature Cited

1. Rebeiz, C. A.; Montazer-Zouhoor, A.; Hopen, H. J.; Wu, S. M. *Enzyme Microb. Technol.* **1984**, *6*, 390-401.
2. Lydon, J.; Duke, S. O. *Pestic. Biochem. Physiol.* **1988**, *31*, 74-83.
3. Matringe, M.; Scalla, R. *Plant Physiol.* **1988**, *86*, 619-22.
4. Witkowski, D. A.; Halling, B. P. *Plant Physiol.* **1988**, *87*, 632-7.
5. Beale, S. I.; Weinstein, J. D. in *Biosynthesis of Heme and Chlorophylls*, Dailey, H. A. Ed.; McGraw-Hill, New York), **1990**, pp. 287-391.
6. Rebeiz, C. A.; Montazer-Zouhoor, A.; Mayasich, J. M.; Tripathy, B. C.; Wu, S. M.; Rebeiz, C. C. *CRC Crit. Rev. Plant Sci.* **1988**, *6*, 385-436.
7. Rebeiz, C. A.; Reddy, K. N.; Nandihalli, U. B.; Velu, J. *Photochem. Photobiol.* **1990**, *52*, 1099-1117.
8. Rebeiz, C. A.; Nandihalli, U. B.; Reddy, K. N. in *Herbicides*, Baker, N. R.; Percival, M. P., Eds.; Elsevier Science Publishers: New York, **1991**; pp. 173-208.
9. Duke, S. O.; Becerril, J. M.; Sherman, T. D.; Lydon, J.; Matsumoto, H. *Pestic. Sci.* **1990**, *30*, 367-78.
10. Duke, S. O.; Becerril, J. M.; Sherman, T. D.; Matsumoto, H. *Am. Chem. Soc. Symp. Ser.* **1991**, *449*, 371-86.

11. Duke, S. O.; Lydon, J.; Becerril, J. M.; Sherman, T. D.; Lehnen, L. P.; Matsumoto, H. *Weed Sci.* **1991**, *39*, 465-73.
12. Scalla, R.; Matringe, M.; *Z. Naturforsch.* **1990**, *45c*, 503-11.
13. Granick, S. *J. Biol. Chem.* **1961**, *236*, 1168-72.
14. Duggan, J.; Gassman, M. *Plant Physiol.* **1974**, *53*, 206-15.
15. Chakraborty, N.; Tripathy, B. C. *Plant Physiol.* **1992**, *98*, 7-11.
16. Duggan, J.; Gassman, M. *Plant Physiol.* **1979**, *64*, 393-7.
17. Ryberg, M.; Sundqvist, C. *Physiol. Plant.* **1976**, *36*, 356-61.
18. Bednarik, D. P.; Hoober, J. K. *Archiv. Biochem. Biophys.* **1985**, *240*, 369-79.
19. Nandihalli, U. B.; Rebeiz, C. A. *Pestic. Biochem. Physiol.* **1991**, *40*, 27-46.
20. Nandihalli, U. B.; Duke, M. V.; Duke, S. O. *Pestic. Biochem. Physiol.* **1992**, In press.
21. Nandihalli, U. B.; Duke, M. V.; Duke, S. O. *J. Agric. Food Chem.* **1992**, In press.
22. Mostowska, A.; Kittsteiner, U.; Rudiger, W. *Protoplasma.* **1991**, *161*, 23-30.
23. Mostowska, A.; Fiett, D.; Parys, E. *Environ. Expt. Bot.* **1991**, *31*, 385-95.
24. Hodgins, R. R. W.; van Huystee, R. B. *Physiol. Planta.* **1989**, *76*, 333-9.
25. Rebeiz, C. A.; Wu, S. M.; Kuhadja, M.; Daniell, H.; Perkins, E. J. *Mol. Cell. Biochem.* **1983,** *57*, 97-125.
26. Averina, N. G.; Shalygo, N. V.; Yaronskya, E. B.; Rassadina, V. V. *Fiziologiya Rastenii*, **1988**, *35*, 679-86.
27. Askira, Y.; Rubin, B.; Rabinowich, H. D. *Free Radical Res. Commun.* **1991**, *12-13*, 837-43.
28. Nandihalli, U. B.; Liebl, R. A.; Rebeiz, C. A. *Pestic. Sci.* **1991**, *31*, 9-23.
29. Matringe, M.; Scalla, R. *Pestic. Biochem. Physiol.* **1988**, *32*, 164-72.
30 Duke, S. O.; Lydon, J.; Paul, R.N. *Weed Sci.* **1989**, *37*, 152-60.
31. Yanase, D.; Andoh, A. *Pestic. Biochem. Physiol.* **1989**, *35*, 70-80.
32. Hayashi, Y.; Furusaki, A.; Tagashira, M. *J. Agric. Food Chem.* **1990**, *38*, 2086-9.
33. Lyga, J. W.; Patera, R. M.; Theodoridis, G.; Halling, B. P.; Hotzman, F. W.; Plummer, M. J. *J. Agric. Food Chem.* **1991**, *39*, 1667-73.
34. Lyga, J. W.; Halling, B. P.; Witkowski, D. A.; Patera, R. M.; Seeley, J. A.; Plummer, M. J.; Hotzman, F. W. *Amer. Chem. Soc. Symp. Ser.*, **1991**, *443*, 170-81.
35. Mito, N.; Sato, R.; Miyakado, M.; Oshio, H.; Tanaka, S. *Pestic. Biochem. Physiol.* **1991**, *40*, 128-35.
36 Morishima, Y.; Osabe, H.; Goto, Y. *J. Pestic. Sci.* **1990**, *15*, 553-9.
37. Sherman, T. D.; Duke, M. V.; Clark, R. D. ; Sanders, E. F; Matsumoto, H.; Duke, S. O. *Pestic. Biochem. Physiol.* **1991**, *40*, 236-45.
38. Van Saun, W. A.; Bahr, J. T.; Crosby, G. A.; Fore, Z. Q.; Guscar,H. L.; Harnish, W. N.; Hooten, R. S.; Marquez, M. S.; Parrish, D. S.; Theodoridis, G.; Tymonko, J. M.; Wilson, K. R.; Wyle, M. J. *Brit. Crop Prot. Conf.-Weeds*, **1991**, *2-9*, 77-82.
39. Nandihalli, U. B.; Sherman, T. D; Duke, M. V;Fischer, J. D.; Musco, V.A.; Becerril, J. M.; Duke, S.O. *Pestic. Sci.* **1992**, In press.
40. Matringe, M.; Camadro, J. M.; Labbe, P.;Scalla, R. *FEBS Lett.* **1989**, *245*, 35-8.
41. Witkowski, D. A.; Halling, B. P. *Plant Physiol.* **1989**, *90*, 1239-42.
42. Jacobs, J. M.; Jacobs, N. J.;Borotz, S. E.; Guerinot, M. L. *Arch. Biochem. Biophys.* **1990**, *280*, 369-75.

43. Jacobs, J. M.; Jacobs, N. J.; Sherman, T. D.; Duke, S. O. *Plant Physiol.* **1991**, *97*, 197-203.
44. Lehnen, L. P.; Sherman, T. D.; Becerril, J. M.; Duke, S. O. *Pestic. Biochem. Physiol.* **1990**, *37*, 239-48.
45. Matringe, M.; Camadro, J. M.; Block, M. A.; Joyard, J.; Scalla, R.; Labbe, P.; Douce, R. *J. Biol. Chem.* **1992**, *267*, 4646-51.
46. Jacobs, J. M.; Jacobs, N. J. *Arch. Biochem. Biophys.* **1987**, *244*, 219-24.
47. Sherman, T. D.; Becerril, J. M.; Matsumoto, H.; Duke, M. V.; Jacobs, J. M.; Jacobs, N. J.; Duke, S. O. *Plant Physiol.* **1991**, *97*, 280-7.
48. Becerril, J. M.; Duke, S. O. *Plant Physiol.* **1989**, *90*, 1175-81.
49. Becerril, J. M.; Duke, S. O. *Pestic. Biochem. Physiol.* **1989**, *35*, 119-26.
50. Matsumoto, H.; Duke, S. O. *J. Agric. Food Chem.* **1990**, *38*, 2066-71.
51. Becerril, J. M.; Duke, M. V.; Nandihalli, U. B.; Matsumoto, H.; Duke, S. O. *Physiol. Planta.* **1992**, In press.
52. Watanabe, H.; Ohori, Y.; Sandmann, G.; Wakabayashi, K.; Boger, P. *Pestic. Biochem. Physiol.* **1992**, *42*, 99-109.
53. Camadro, J. M; Matringe, M; Scalla, R; Labbe, P. *Biochem. J.* **1991**, *277*, 17-21.
54. Varsano, R.; Matringe, M.; Magnin, N.; Mornet, R; Scalla, R. *FEBS Lett.* **1990**, *272*, 106-8.
55. Hayashi, Y. *J. Agric. Food Chem.* **1990**, *38*, 839-44.
56. Jordan, P. M.; Seehra, J. S. *J. C. S. Chem. Comm.* **1980**, *1243*, 240-2.
57. Huault, C.; Blondel, J-D.; Balange, A-P. *Plant Physiol. Biochem.* **1990**, *28*, 137-40.
58. Prakash, T. R.; Swamy, P. M.; Prasad, D. D. K. *Pestic. Biochem. Physiol.* **1990**, *36*, 29-34.
59. Shioi, Y; Doi, M; Boddi B. *Arch. Biochem. Biophys.* **1988**, *267*, 69-74.
60. Wong, K. K. *Plant Sci. Lett.* **1978**, *13*, 269-73.
61. Poulson, R.; Polglase, W. J. *Biochem. Biophys. Acta*, **1973**, *329*, 256-63.
62. Smith, A. G. *Biochem. J.* **1988**, *249*, 423-8.

RECEIVED July 22, 1992

Chapter 6

Perspectives on Providing a Realistic Technical Foundation for the Commercialization of Bioherbicides

P. S. Zorner, S. L. Evans, and S. D. Savage

Mycogen Corporation, 5451 Oberlin Drive, San Diego, CA 92121

Biological weed control has been an active research area for several decades, but certain key technical barriers have prevented broad commercial adoption of this technology. The last 10 years have been a productive decade for the discovery of new microbial herbicides in that over 100 pathogens have been described in the literature as having potential as biocontrol agents, and a recent survey has identified 200 scientists working in this area. Unfortunately, only two pathogens have been developed to the point of commercialization. A commercial product is defined as one that works consistently in a field environment, and that also can be economically produced and formulated in a form that will maintain the viability of the organism through a commercial distribution process. It appears that limitations to development of additional commercial products are not due to the failure to discover pathogens or due to insufficient personnel involved in research. We conclude that commercialization of additional bioherbicides depends upon devoting major efforts toward developing appropriate fermentation, stabilization and delivery technology.

The use of classical synthetic herbicides is becoming more complicated for industry and their customers as legislative and efficacy issues arise. Weed resistance is limiting efficacy (1). Chemical residues are being found in ground water (2). Persistence of some herbicides beyond a single growing season affects rotational crops (3). Environmental and social concerns are causing some chemicals to be taken out of smaller cropping segments as industry finds these markets cannot financially support the costs of reregistration. These issues and others are making modern weed management a complex process that goes far beyond simple efficacy issues.

Biological herbicides may offer some relief to these problems. Generally, they have low registration costs. Mycogen estimates these costs to be no more than

0097–6156/93/0524–0079$06.00/0

one million dollars for naturally occurring biological herbicides. The fixed costs for production are low because it is not normally necessary to build a specialized manufacturing facility for these materials. Plenty of excess fermentation capacity exists in the world to handle fermentation of bioherbicides. Bioherbicides have a positive public image that is associated with the overall perception of biological pest control. Bioherbicides also offer a killing action that is different from synthetic herbicides, so they should be efficient tools in combating weeds resistant to specific chemicals. However, despite these obvious beneficial traits, the technology has not realized broad commercial utility even though the technology has been under development for over 25 years (4).

Current Scientific Effort on Bioherbicides

The lack of success in developing this technology does not appear to be due to a lack of scientific effort or effective microorganisms. Charudattan reports that in 1989 mycoherbicide research was being conducted in 44 different locations across 16 different countries (4). A recent survey for the Weed Science Society of America identified over 200 scientists in North America working to some extent on biological weed control (Zorner, P.S., Mycogen Corporation, unpublished data). These people have been relatively successful in finding effective microorganisms. In a recent survey, Charudattan listed over 109 pathogens that have been in various stages of development over the last couple of decades (4). In the last 10 years 73 organisms were placed into commercial development programs (4). However, only two of these organisms have been commercialized, and there have been no commercial introductions in the last ten years. Despite this lack of commercial success, Charudattan reported that about 10 percent of the persons surveyed reported that commercial use of the organisms they were working on was considered to be "imminent" (about 9 organisms).

The optimistic projections create the impression that weed biocontrol technology has recently overcome major commercial limitations and that people are very confident in their current projects. However, this does not seem to be the case. However, Charudattan also found that over 60% of the more than 100 scientists that he polled felt the chances for commercial success of the pathogen they were working with were less than fair or had unknown chances of success. It appears that one basic problem facing this technology is that basic researchers do not understand what is needed to successfully commercialize a biological product and therefore, do not focus their energy and resources on resolving the key technical issues. In any event, it appears that a strong technical foundation for commercial success of biological weed control has not yet been achieved, despite the efforts of many people and their evident success in finding microorganisms capable of killing plants.

Factors Important to Establishing a Commercial Product

Unfortunately, providing a solid technical foundation for effective commercial utility involves much more than just finding microorganisms capable of killing weeds. It also requires developing formulations that allow them to work consistently under diverse field conditions. It requires an ability to stabilize these living organisms to the point that they can survive a distribution process in which the formulated product may remain in a warehouse for as long as 18 months between packaging and use. It requires showing that the plant pathogens that form the base of the commercial weed control product do not pose an environmental threat to other plants or segments of the ecosystem in which they will be used.

Addressing all aspects of this commercial foundation is a great deal of work and many people feel that biological weed control is severely resource limited, and thereby cannot address all the critical issues. It is also our opinion that the science of biological weed control is not resource limited, but is concept and/or idea limited i.e., that as a group we don't fully understand what technical barriers are limiting our progress. It does not seem reasonable to blame a lack of commercial success on a lack of resources when the last 10 years has seen the discovery of 73 developmental leads (4), and when 200 people in North America alone indicate they are working in this area (Zorner, P.S., Mycogen Corporation, unpublished data). The fact that 73 developmental leads have been found and never commercialized indicates that too much effort has been put on finding new organisms, and not enough effort has been placed on developing the technology to address the other important parts of the commercial foundation mentioned above, such as obtaining consistent efficacy under field conditions and providing prolonged stability of a live organism in a commercial formulation.

Recognizing Basic Biological Principals

Reviewing the classical disease triangle allows some insight into the nature of the problem of obtaining consistent field efficacy with a living plant pathogen (5). The onset of disease in a function of having a viable pathogen in contact with the correct plant host under environmental conditions that are required for the pathogen in question. The whole foundation of biological weed control is to push the disease process by tipping the ecological balance in favor of the pathogen. People have generally done this by looking for the "right" pathogen or one that will work in a diversity of environments. Thus, we get 73 new organisms as people look for that one organism that will work consistently in the market they want to address. However, the key to the process is not to concentrate on the pathogen itself, but to tip the ecological balance in favor of the pathogen being used by applying it in a viable form, at inoculum levels high enough to initiate an infection and trying to manipulate the micro-environment for a long enough period of time to make sure that an infection gets to the point that it can perpetuate itself.

Progress won't be made by serving a research philosophy that is based in the concept that if we screen enough organisms, we will eventually find a few unique pathogens that have all the inherent properties necessary to support a commercial product. A greater level of effort is needed in developing technology that can modify the micro-environment and/or enhance the survival of organisms selected to function in a weed control market that is well-suited for a biological organism.

The problem in obtaining even an initial infection with the current leads summarized by Charudattan is that they are all fungal pathogens (4). The underlying technical problem with these fungi is that infection requires some period of dew on the surface of the plant so that the fungal spore can germinate and infect the plant. The dew periods of known bioherbicides can approach 72 hrs and are at least 8 hrs (6,7). Unfortunately, few natural environments provide more than a few hours of dew, and as summarized by Charudattan, field tests of these fungi demonstrate a great deal of inconsistency (8).

The situation is not unlike planting crop seed. Seed is seldom scattered on fallow land and left to germinate and initiate a crop without any further efforts to manipulate the process. Weeds are generally removed, the soil prepared, the seed placed at a soil depth that provides some consistency in moisture, some fertilizer placed with the seed and even a few pesticides applied to give the seed an advantage over indigenous pathogens, insects and weeds. A fungal bioherbicide spore also requires manipulation. It will infect and kill the plant if it is viable and is given the appropriate environmental stimulus to carry out its life cycle. It will die if these environmental requirements are not met. A few people are working on these problems (9). Unfortunately, this technology has not been developed to the point that it economically allows appropriate manipulation of the micro-environment, and thus most applications of bioherbicides in the field provide little or no help to the organism in serving the agricultural function it is being asked to provide. However, people continue to search for new fungal pathogens and test their efficacy under field conditions. In some instances people have chosen organisms for which it is hard to imagine any technology providing the necessary environmental assistance, such as a fungal pathogen that requires a 48 hr dew period (7). Working with an organism of this type beyond the stage of initial discovery makes little sense when no known commercial technology exists that will economically and consistently improve the field performance of an organism, such as *Alternaria cassaie*, that has only an 8 hr dew period.

Bacteria as Bioherbicides

It has recently been shown that it is also possible to consider the use of bacteria as biological control agents. Thus, it may be possible to escape the limitations of a dew requirement. *Xanthomonas campestris* pv. *poannua* is being developed as a biocontrol for *Poa annua* in turf (10). It has been shown not to require a dew period because the infection process occurs through movement of the bacteria into the host via a direct wound introduced by a mowing operation. Field efficacy was shown to be quite consistent in the southeastern United States. However, disease development and plant death required as long as 50 to 60 days. The researchers involved reported no dew period requirements for disease progression, but did report a strong degree-day requirement (11). This organism requires about 800 degree days above a baseline of 3°C in order to provide 80% weed control. This is a prolonged period of warm temperatures and may prove to be as important a limitation in cooler climates as a dew period limitation is for various fungi.

A point of interest here is that bacteria can be used as effective biocontrol agents, and that they may allow people to develop effective bioherbicides in the absence of technology to resolve dew period requirements. However, environmental limitations on efficacy are not avoided with bacteria. They simply have been transformed from a dew period requirement into a temperature requirement, and a requirement for some form of mechanical wounding to allow penetration. Since all bioherbicides are living organisms, it is likely that all potential pathogens will carry some sort of environmental limitation on efficacy and this is a fact that must be dealt with in any discovery program.

Choosing the Correct Target

Recognizing that environmental limitations cannot be avoided is an important part of any discovery program, because it puts a greater emphasis on appropriate target selection. "Target" is being used here to represent not only the weed species being selected for control, but the cropping system in which that weed is a problem. Logical bioherbicide targets are normally considered to be weeds which escape chemical controls, weeds which escape cultural controls, weeds in environmentally sensitive settings, and weeds in organic cropping systems. In practice, the ideal target is a weed in an agricultural system that may allow for some environmental modification and where people have an economic incentive to make those modifications. Thus, commercial development of bioherbicide candidates into markets such as corn or dry land wheat make much less sense than targets such as turf or high value fruits and vegetables, especially in the absence of technology to manipulate the micro-environment through the formulation process. The logic behind this statement is based on the opinion that corn or wheat are large enough markets to support registration of new, effective and reasonably priced chemicals. Bioherbicide technology would have a difficult time competing on an economic basis and there is little incentive to a grower to use these products at this time. But probably more important is the fact that these and other row crops are grown in a diversity of environments and prolonged dew periods or the ability to manufacture them would be rare. Consistent efficacy would be a problem. Turf, on the other hand, is generally located in environmentally sensitive locations where people have major concerns about pesticide use, irrigation or mowing is a part of the management system, and thus would not be a limitation if needed to move a pathogen into the weed. This is not to say bioherbicides won't eventually be used in row crops. The point is that other markets make more sense for initial introduction of a technology that is still in its infancy. In summary, a productive discovery effort not only turns out active pathogens, but turns out pathogens that are well suited for the market in which they will ultimately be used and which are well suited for the delivery technology which exists at the time.

Other Key Technical Barriers

As discussed previously, regulatory, fermentation and delivery issues are also major barriers to developing effective commercial bioherbicides. Regulatory, however, is not so much of a technical barrier as it is recognizing that these plant pathogens must be shown to pose no significant risk to the environment in which they will be released. The process of doing this is somewhat confusing because no set regulatory guidelines for doing this have been published by the federal government. The process is basically dependent on a strong and continual interaction with the federal Animal and Plant Health Inspection Service (APHIS). Our experience with APHIS suggests that this process is time consuming, but is not a major technical challenge. Early interaction with APHIS is critical to setting the questions to be answered for any particular organism. These questions generally revolve around host range, environmental distribution, clear identification of the organism and the dynamics of population survival following an inundative release. Technical protocols for providing these data usually exist or are not hard to devise, and thus it is simply a matter of doing the work and communicating on a frequent basis with regulatory authorities.

On the other hand, technology for submerged fermentation, stabilization and delivery of a broad range of living biological herbicides does not exist. Bioherbicides will never be widely used as commercial weed control agents until this technology is developed because it impacts their cost, their ability to maintain viability during the distribution process and their ability to work consistently in a field environment. A review of the current technology has been written by Stowell (12). In our opinion this area represents the major technical limitation to establishing bioherbicides as a commercial reality. Yet, in the survey conducted for the WSSA, only 15 of 200 people who responded to the survey indicated that they were working in this area (Zorner, P.S., Mycogen Corporation, unpublished data). This must change if progress is to be made is resolving the key technical issues.

Fermentation and Stabilization as Essential Issues

The importance of delivery technology was stated previously. Some explanation of why fermentation and stabilization issues are so important also seems appropriate. To do this, consider an imaginary organism produced at a yield of 10^{10} colony forming units (cfu's)/ml or spores/ml in the case of fungi. Furthermore, assume that we obtain 50% recovery of viable propagules through the process that leads from the fermentation broth to a formulated product. Finally, assume that it requires 10^9 cfu's/ml (or spores/ml) delivered in 400 liters/hectare of volume in the field to obtain efficacy, and that the associated costs of production for this product, at this use rate are $10/hectare. The importance of making improvements or losing ground relative to the number of viable propagules can be recognized by looking at how dramatically this $10/hectare figure can change with various losses or gains in the system. For example; if yields fall an order of magnitude during scale-up from small fermentors to tanks, production costs rise to $100/hectare. On the other hand, if yields can be increased by an order of magnitude through a clear understanding of those events that support high density fermentation, the costs of production would fall to $1.00/hectare. These two extremes in cost would have a tremendous effect on a business decision to commercialize a living biological. A similar scenario can be set up for stability of the organism once it is formulated. A stable organism may not lose any viability over a one year storage period. An unstable organism could lose one or even two logs of activity. The costs of overformulating to compensate for this loss could drive the cost per acre from $10 to perhaps $1000/hectare. Obviously, these are all imaginary examples, but the point is that micro organisms operate in log scales and logarithmic changes in viable cell or spore count, or even a portion thereof will have a huge impact on the commercial feasibility of bioherbicides. However, despite the critical nature of these processes and a lack of public information on how to control these processes, fewer than 10% of the people involved in bioherbicide research in North America are focusing on this area. It seems logical to assume that this is why only two commercial organisms exists from a pool of 73 leads placed into development over the last 10 years.

Summary

In summary, it is our opinion that the current lack of commercial bioherbicide products arises from an unnecessary emphasis of current research efforts on finding new organisms. Discovery of active bioherbicides is not the major limitation to developing this technology. Much more effort needs to be placed on developing technology to economically ferment, stabilize and deliver these organisms in a form that will provide for consistent field efficacy. Individual scientists need to form liaisons with people who have these skills and begin to work in multidisciniplinary teams. If these issues are not resolved, they will effectively prevent broad commercial adoption of bioherbicides as effective weed control tools.

Literature Cited

1. Holt, J.S.; LeBaron, H.M. *Weed Technol.* 1990, 4, 141-149.
2. Carter, A.D.; Hollis, J.M.; Thompson, T.R.; Oakes, D.B.; Binney, R. *Brighton Crop Protection Conf., Weeds* 1991, Vol. II, 491-498.
3. Stougard, R.N.; Shea, P.J.; Martin, A.R. *Weed Sci.* 1990, 38, 67-73.
4. Charudattan, R. In *Microbial Control of Weeds*; Editor, D.O. TeBeest; Chapman and Hall: New York, NY, 1991, 24-57.

5. Sharvelle, E. *Plant Disease Control;* AVI Publishing Company: Westport, CN, 1979, 16-34.
6. TeBeest, D.O. In *Microbial Control of Weeds*; Editor, D.O. TeBeest; Chapman and Hall: New York, NY, 1991, 97-114.
7. Connick, A.C.; Dagle, J.S. In *Microbes and Microbial Products as Herbicides*; Editor, R.E. Hoagland; ACS Symposium Series No. 439; American Chemical Society: Washington, DC 1990, 286-304.
8. Charudattan, R. In *Microbes and Microbial Products as Herbicides*; Editor, R.E. Hoagland; ACS Symposium Series No. 439; American Chemical Society: Washington, DC 1990, 132.
9. Boyette, C.D.; Quimby, P.C.; Connick, W.J.; Daigle, D.J.; Fulgham, F.E.In *Microbial Control of Weeds*; Editor, D.O. TeBeest; Chapman and Hall: New York, NY, 1991, 209-222.
10. Haygood, R.A. WSSA Abstracts, 1992, 32, 28.
11. Kalmowitz, K; Monaco, T.; Zorner, P.; Evans, S. WSSA Abstracts, 1992, 32, 50.
12. Stowell, L.J. In *Microbial Control of Weeds*; Editor, D.O. TeBeest; Chapman and Hall: New York, NY, 1991, 225-261.

RECEIVED September 14, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

Chapter 7

Synergizing Weed Biocontrol Agents with Chemical Herbicides

A. L. Christy, K. A. Herbst, S. J. Kostka, J. P. Mullen, and P. S. Carlson

Crop Genetics International, 7170 Standard Drive, Hanover, MD 21076

Enhanced weed control can occur when chemical herbicides are applied in combination with microbial agents. Synergy between chemicals and biologicals was first observed with fungal bioherbicides. Use of a herbicide with a fungal-bioherbicide does not appear to widen the weed spectrum of the bioherbicide but does increase the efficacy. In some cases the synergy may result from a suppression of phytoalexin synthesis by the chemical herbicide. Use of synthetic herbicides to synergize mycoherbicides will in some cases reduce herbicide use rate and improve mycoherbicide efficacy. We report here that synergy occurs between chemical herbicides and bacterial agents. We have discovered that bacteria which cause little or no injury to weeds when applied alone can enhance the stability of low levels of chemical herbicides to control a broad spectrum of weeds. We term this synergistic combination the X-tend system. In the first year of X-tend field trials, the level of weed control required for commercialization was not obtained consistently across geographic locations. Consistency will need to be improved through selection of more robust microbial strains and/or improvements in formulations.

The use of biocontrol agents to control pests in the United States celebrated a 100 year anniversary in 1989. While there are a number of examples of very successful biocontrol projects, the widespread use of biological-based pest control technology has not been adapted to major agricultural production systems.

The growing concern over the continued use of synthetic chemical pesticides has promoted efforts to expand biocontrol technologies for major pest problems. To date, however, biological pest control technologies have not proven as effective or economical as chemical control. Many biocontrol

0097–6156/93/0524–0087$06.00/0

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

products targeted for control of major pests are still in the research and development stage.

Some of the problems with using biocontrol to manage pest problems include: 1) slow and/or insufficient activity, 2) limited pest control spectrum, 3) inconsistent activity across different environments, and 4) high cost of production. One approach to overcome some of these problems may be to combine chemical and biological control agents in an integrated pest management system. Synergy between synthetic chemical and biological agents may result in both a reduction in chemical application rate and an increase in efficacy of the biological agent. Such a system may both reduce the chemical load on the environment and reduce the cost of pest control to the grower.

Several recent studies have reported possible synergy between synthetic chemical herbicides and fungal plant pathogens for use in weed control (1, 2). In addition, we have been developing a weed control system known as X-tend that combines bacterial agents and reduced rates of herbicides to control major agriculturally important weeds.

Fungal-Based Bioherbicides

The majority of the bioherbicides studied to date have involved the use of fungal pathogens to control weeds. Two commercially available bioherbicides, Collego (Colletotrichum gloeosporioides (Penz). Sacc. f. sp. aeschynomene) and DeVine (Phytophthora palmivora) are each specific for only one weed; northern jointvetch, Aeschynomere virginica, and milkweed vine, Morrenia odorata, respectively. In addition, fungal bioherbicides require specific conditions for infection and disease development. For example, a dew period in excess of 24 h is often required for spore germination. Variable environmental conditions may severely hamper effective control.

Some examples of synergy observed between fungal pathogens and herbicides are summarized in Table I. Holliday and Keen (1) were perhaps the first to demonstrate that a herbicide could block the expression of resistance to a fungal pathogen. Treating soybean plants with glyphosate appeared to promote infection by an incompatible race of the fungus Phytophthora megasperma f. sp. glycinea (1, 3).

These data suggest that compromising the plant in some way with a chemical agent reduces the plant's ability to mount a defense to pathogen attack and improves the efficacy of the bioherbicide. Keen et al. (3) found that low levels of glyphosate blocked production of glyceollin, the phytoalexin in soybean thought to provide some resistance to Phytophthora megasperma infection. While an undesirable effect in a crop, it is a desirable synergy in weed control. For example, Sharon et al. (4) demonstrated that glyphosate inhibited phytoalexin synthesis in treated sicklepod, possibly reducing plant resistance to fungal attack and enhancing the activity of the bioherbicide Alternaria cassiae.

Table I. Some Examples of Synergistic Fungal Pathogen and Chemical Combinations

Pathogen	Herbicide/PGR	Plant	Reference
Phytophthora megasperma	glyphosate	soybean	Keen et al., 1982 (3)
Colletotrichum coccodes	thidiazuron	velvetleaf	Wymore et al., 1987 (3) Hodgson et al., 1988 (8)
Alternaria cassiae	numerous herbicides	several	Caulder and Stowell, 1988 (9)
Fusarium lateritiun	bentazon and acifluorfen	Florida beggarweed	Caulder and Stowell, 1988 (9)
Pythium and Fusarium ssp.	glyphosate	black bean	Rahe et al., 1990 (10)
Alternaria cassiae	glyphosate	sicklepod	Sharon et al., 1991 (4)
Alternaria cassiae	invert emulsion	sicklepod	Amsellem et al., 1990 (11)

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

Two research groups have reported synergy between the fungus Colletotrichum coccodes and the plant growth regulator (PGR) thidiazuron for the control of velvetleaf (Abutilon theophrasti) (5, 6). Wymore et al. (5) showed that separate applications of C. coccodes and thidiazuron reduced velvetleaf growth and provided some level of weed control. However, application of tank mixes of thidiazuron and C. coccodes spores resulted in as much as 100% mortality of velvetleaf in both greenhouse (5) and field studies (7). Hodgson et al. (8) confirmed these results in field studies in both Canada and Maryland (Figure 1). The mode of action of the synergy between thidiazuron and C. coccodes was investigated by Hodgson and Synder (6). Application of thidiazuron or an extract of C. coccodes mycelium to velvetleaf petioles enhanced ethylene production. However, it is not clear from this research whether the ethylene was produced as a result of thidiazuron application. The increased ethylene level may contribute to the observed synergy or may be a response of the plant tissue to the application of the PGR (6).

Caulder and Stowell (9) received a US patent in 1988 for the use of four fungal plant pathogens in combination with a number of herbicides and PGR's. Table II summarizes the synergy they observed with various fungal and chemical combinations. It is interesting to note that the chemical agents represent a variety of chemical classes with different modes of action. Synergy was observed with the fungal pathogen on the target (susceptible)

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

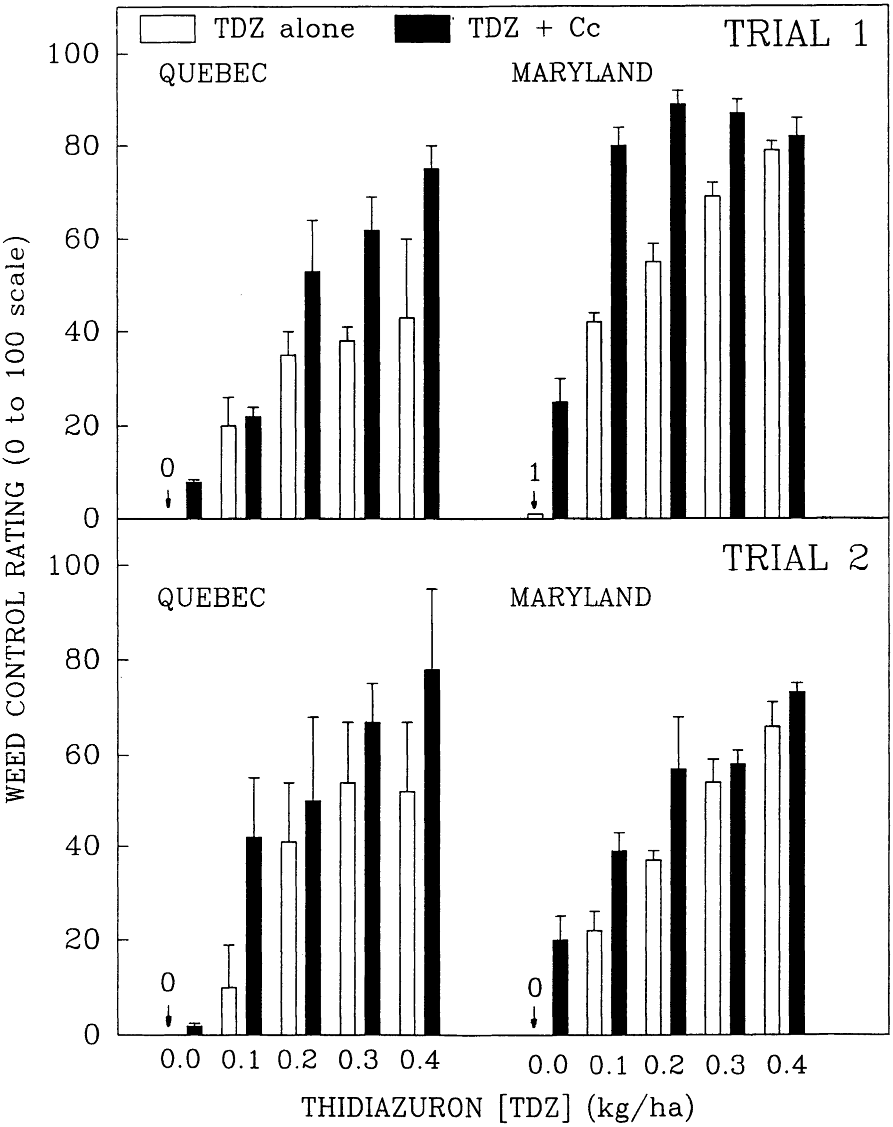


Figure 1. Control ratings for velvetleaf tested with thidiazuron (TDZ) and Colletotrichum coccodes (Cc), Trial 1 treated at 1 to 2 leaf stage, Trial 2 treated at 4 to 6 leaf stage. (Reproduced with permission from reference 6. Copyright 1989, Weed Science Society of America.)

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

Table II. Summary of Synergy Between Four Fungal Pathogens and a Series of Chemical Agents [a]

	Synergy with[b]			
Herbicide/PGR	AC[c]	CC[c]	CT[c]	FL[c]
bentazon	+	+	+	+
acifluorfen	+	+	+	+
chlorimuron	-	+	-	
fluazifop	+		+	
diolofop	+		-	
sethoxydim	+		-	
imazaquin	+		+	
metribuzin	+		-	
oryzalin	+		+	
thidiazuron	-	+	+	
diaminozide	-		+	
mefluidide	+		+	

[a] From US Patent 4,775,405, to J.D. Caulder and L. Stowell (Mycogen Corp.), 1988.

[b] "+" indicates synergy, "-" indicates a lack of synergy, and a blank indicates that the combination as not tested.

[c] AC = Alternaria cassiae
CC = Colletotrichum coccodes
CT = Colletotrichum truncatum
FL = Fusarium lateritium

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

host, however, the herbicide did not expand the weed spectrum of the biocontrol fungus (9).

Sharon et al. (4) showed that glyphosate suppressed the accumulation of total phenolic compounds, flavonoids, and the resulting phytoalexins in sicklepod (Cassia obtusifolia L.) (Figure 2). This resulted in glyphosate-treated sicklepod seedlings being highly susceptible to the fungal pathogen Alternaria cassiae (4). However, when soybean plants were treated with glyphosate, no increase in susceptibility to A. cassiae was observed. These results are consistent with earlier reports in which herbicide-mycoherbicide combinations did not expand the host range of the mycoherbicide.

Other herbicides and PGR's have been reported to synergize fungal pathogens. The role of these herbicides and PGR's on the inhibition of phenolics, flavonoids, and phytoalexins has not been investigated at this time. Perhaps chemicals that stress the plant and reduce growth and metabolism may also reduce phytoalexin synthesis either directly or indirectly and synergize the bioherbicide. It is equally plausible that other physiological processes may be influenced and yield similar phenomenology.

Bacteria-Based Bioherbicides

Research on bacteria as bioherbicides has focused primarily on the use of rhizobacteria (12, 13, 14). Kennedy et al. (12) characterized rhizosphere strains of fluorescent pseudomonads that, when applied to soils, inhibited the germination and growth of downy brome (Bromus tectorum) without affecting the growth of wheat. Similarly, Harris and Stahlman (15) identified rhizosphere strains that inhibited root elongation in one or more grasses without significantly affecting winter wheat root growth. Rhizobacteria representing diverse gram-negative bacterial genera have been demonstrated by Kramer et al. (13, 14) as potential biocontrol agents for broadleaf weeds. Although examining expanded host ranges within either monocots or dicots, none of the researchers screened candidate strains against both groups of plants.

A novel approach to control of annual bluegrass through the exploitation of a vascular, phytopathogenic bacterium is under development. (16). Xanthomonas campestris, the causal agent of bacterial wilt of annual bluegrass (Poa annua L.) is applied in the early spring to newly mown grass. The bacterium rapidly colonizes the xylem; reaching populations of up to 1×10^{10} colony forming units(cfu)/ml of tissue, the foliage wilts and the plants die within six weeks. This strain is being developed for use in controlling bluegrass in turf and does not harm desirable turf species.

Another novel approach, combinations of bacterial and chemical agents for enhanced weed control, the X-tend bioherbicide system, is under development by our Company. Research has involved both greenhouse evaluations and field trials with a range of bacteria and herbicide combinations. Bacteria with X-tend activity are representative of a range of well characterized genera and species, as well as, several unidentified plant-associated strains. A summary of this research is included here as an

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

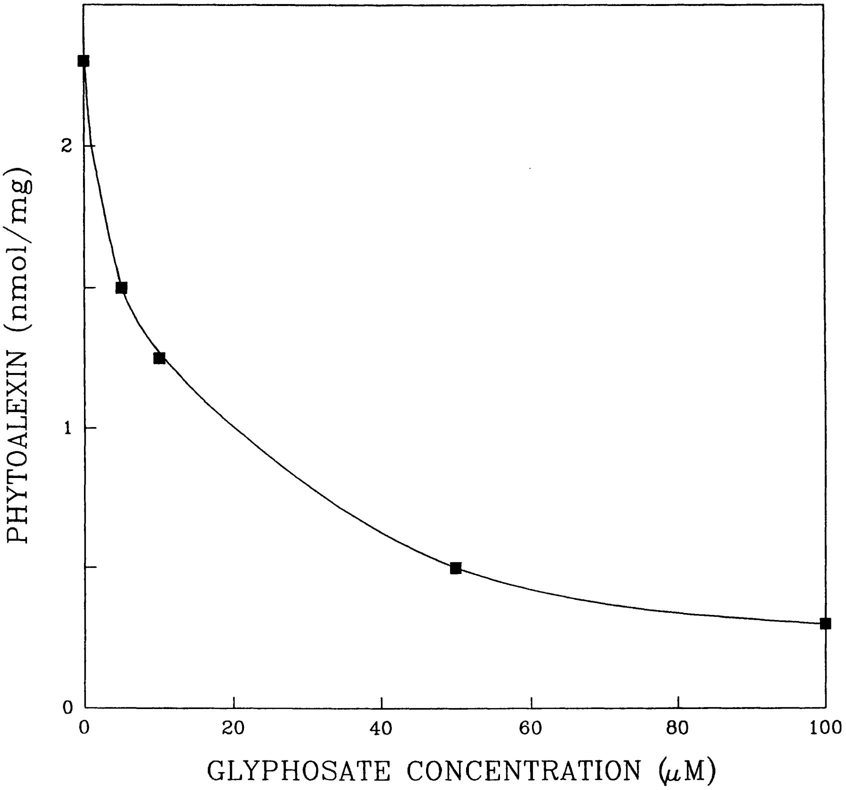


Figure 2. Glyphosate suppression of phytoalexin accumulation in detached leaves of sicklepod (Cassia tectorum). (Reproduced with permission from reference 4. Copyright 1992, American Society of Plant Physiologists.)

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

example of synergy between bacterial agents and synthetic chemical herbicides. All bacteria are referred to by either a strain number or preparation number.

Preparation of Bacterial/Herbicide Tank Mixes. Strains of bacteria were obtained from commercial culture collections, academic and governmental research laboratories, or from isolations made from plant and environmental samples collected throughout the continental United States. Strains were archivally stored by lyophilization and in glycerol (-80°C). Working stocks of specific strains were stored in glycerol at -20°C. Bacteria for plant treatments were produced by growing strains of interest in solid phase or liquid fermentations using one or more culture media incubated at 28°C for 24 to 26 h. Solid phase fermentations were prepared in Petri dishes by streaking the surface of a semi-solid culture medium with a sterile swab soaked with cells from a log phase broth culture.

Liquid fermentations were normally conducted in 2 liter, triple baffled, culture flasks containing 1 liter of broth medium and seeded with bacteria from log phase broth cultures. Depending on the strain, cultures were incubated for 48 to 64 h at 20°C, 25°C, or 28°C on a rotary shaker (180 to 190 rpm).

Cells were harvested from Petri dishes with a rubber policeman or from liquid culture by centrifugation using a Sorval RC5C centrifuge at 8000 rpm (10°C) for 8 to 10 min. Spray solutions were prepared by resuspending cell pellets in either decanted, spent fermentation medium, or sterile distilled water. Cell concentrations were standardized spectrophotometrically (600 nm) to approximately 1×10^9 cfu/ml.

Spray solutions were prepared by adding the chemical herbicides and appropriate surfactant directly into the bacterial preparations. Treatments were applied using a Devries SB-8 spray booth equipped with a 8004E flat fan spray nozzle traveling at 80.5 m/min delivering 233.7 l/ha. One replication consisted of four broadleaf species at the 2 to 3 true leaf stage and four narrowleaf species 8 to 10 cm in height grown in a spaghnum peat: vermiculite: bark ash mix. A randomized complete block design was used with four replications.

Field treatments were applied using a CO_2 pressurized backpack sprayer equipped with 8004 flat fan nozzles delivering 233.7 l/ha at 93.9 m/min. Field studies typically included three to four broadleaf weed species having 4 to 6 true leaves and three to four narrowleaf species 5 to 10 cms in height. Plots were 1.5 m x 3 m with 25 cm row spacing.

Greenhouse Screens. Numerous pathogenic and nonpathogenic bacteria have been evaluated with several herbicides for weed control in the greenhouse. Figure 3 shows the response typically observed with sulfosate (Trimethylsulfonium-carboxymethylamino-methyl phosphonate) applied alone and in combination with a bacterial preparation. Unlike mycoherbicidal fungi, the bacterial preparation applied alone caused no injury to the test weeds. However, the bacterial preparation plus 0.067 kg

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

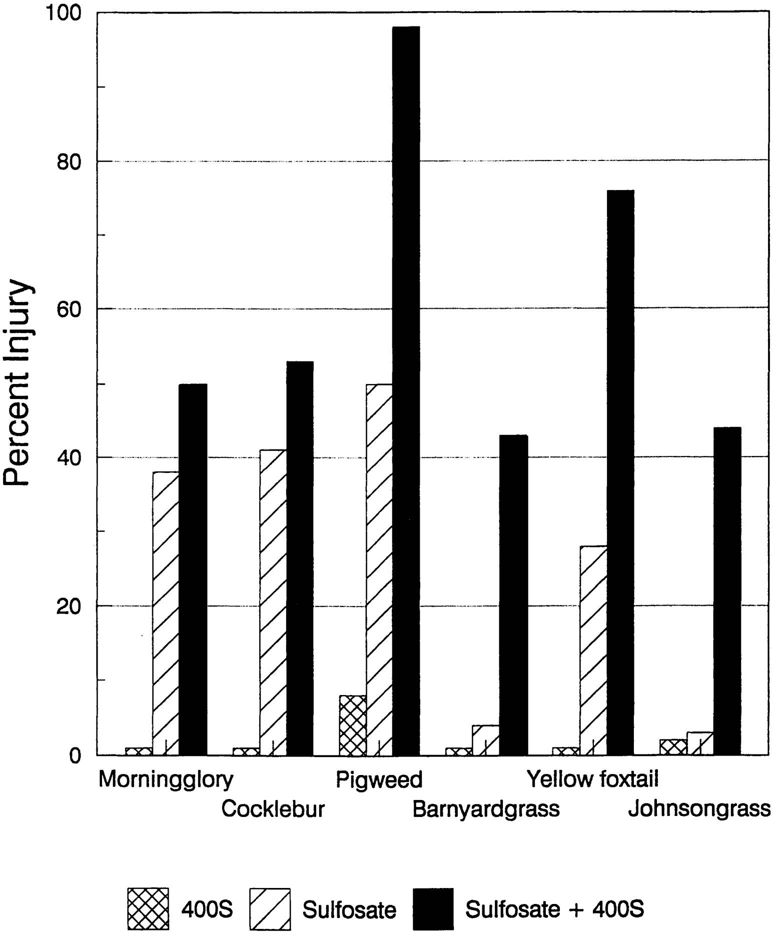


Figure 3. Results of a typical greenhouse screen showing the synergy between sulfosate 0.067 kg ai/ha and a bacterial preparation.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

ai/ha sulfosate demonstrated greater injury than the herbicide alone. Moderate to good control of morningglory (Ipomoea hederacea L.) and pigweed (Amaranthus retroflexus L.), two difficult to control weeds, was observed. The level of injury on narrowleaf weeds was more than twice that of the herbicide alone. Since no injury was observed in treatments where the bacteria was applied alone, the dramatic increase in activity observed with the bacteria-herbicide combination demonstrates synergy.

Field Trials. Maryland field trials of the X-tend bioherbicide preparation 400S plus 0.56 kg ai/ha sulfosate demonstrated a 40 to 50 percentage point increase in control of pigweed, morningglory, and velvetleaf (Abutilon theophrasti) over treatments that received only sulfosate (Figure 4). Preparations of 1518S and 361S in combination with sulfosate improved broadleaf weed control but were less effective than 400S. No synergistic effect on grasses was observed in this study due to complete control by the 0.56 kg ai/ha rate of sulfosate alone.

Similar field results were obtained with glufosinate and two bacterial preparations (Figure 5). Most of the synergy was observed at a rate of 0.224 kg ai/ha glufosinate. This is approximately 1/4 to 1/5 the recommended field rate on the proposed glufosinate label.

Other field trials at our Maryland research farm provided similar results with the herbicides fluazifop, sethoxydim, and nicosulfuron. In most cases as the herbicide rate approached the recommended field rate less synergy with the bioherbicide was observed.

Field trials at other locations revealed a similar pattern (Figure 6). Application of sulfosate at 0.28 kg ai/ha in combination with preparation 400S was synergistic on barnyardgrass at three locations. The herbicide rate was one third the recommended rate and obviously below the rate needed to provide consistent control of barnyardgrass (Echinochloa crus-galli L.). This was particularly evident in tests in Indiana (IN) and Kansas (KS), where the weeds were larger or were grown under drought conditions (IN).

It appears that the bioherbicide was strongly dependent on the level of control or injury provided by the chemical herbicide. The results indicate that a more robust strain of the bacteria will be required to obtain commercial levels of control. Development of surfactant and formulation technology may also aid in improving control.

Summary. Synergy has been observed between chemical herbicides and bacterial agents in both greenhouse and field trials. Combinations of herbicides and bacteria can significantly reduce herbicide use rate to control a broad spectrum of weed species. However, the present level of control achieved with these tank mixes in the field indicates that either more robust strains or improved formulations will be needed before commercialization is realized.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

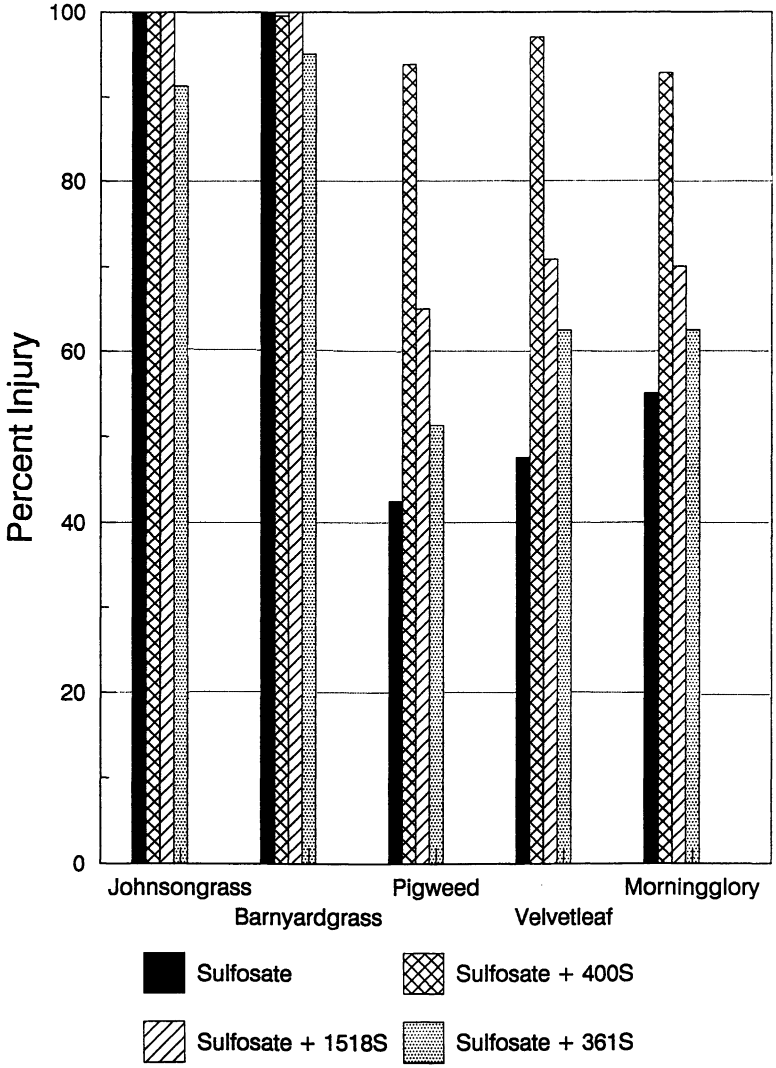


Figure 4. Field study of synergy between sulfosate (0.56 kg ai/ha) and three bacterial preparations.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

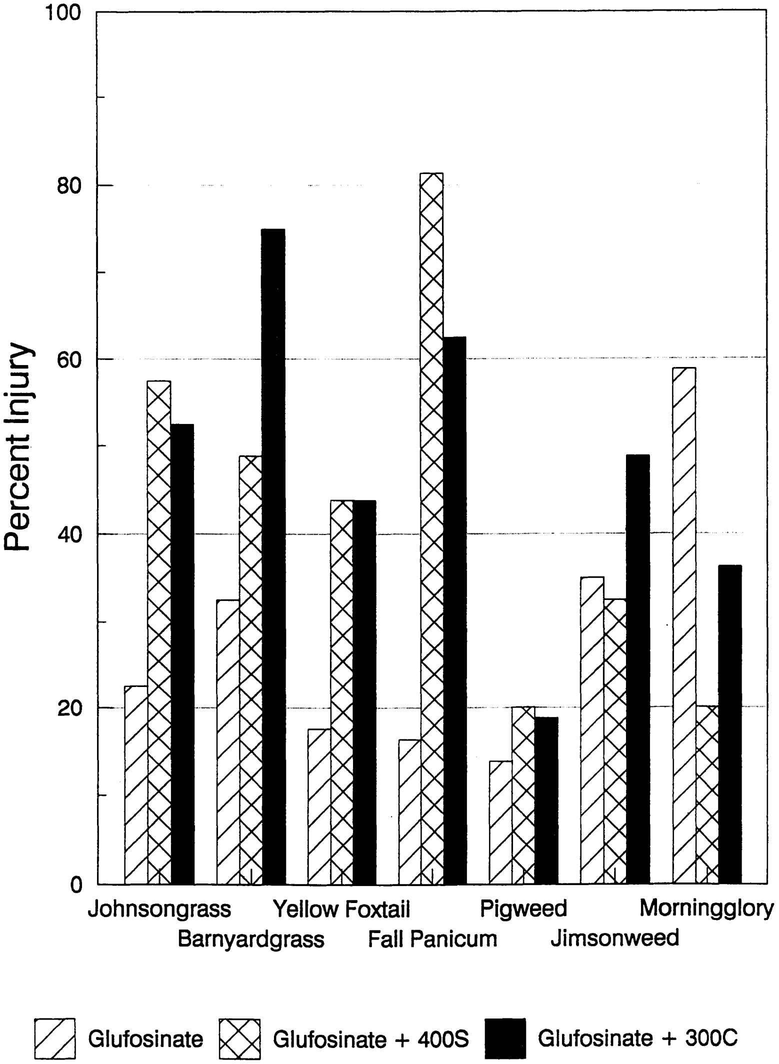


Figure 5. Synergy between glufosinate (0.28 kg ai/ha) and two bacterial preparations in field trials.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

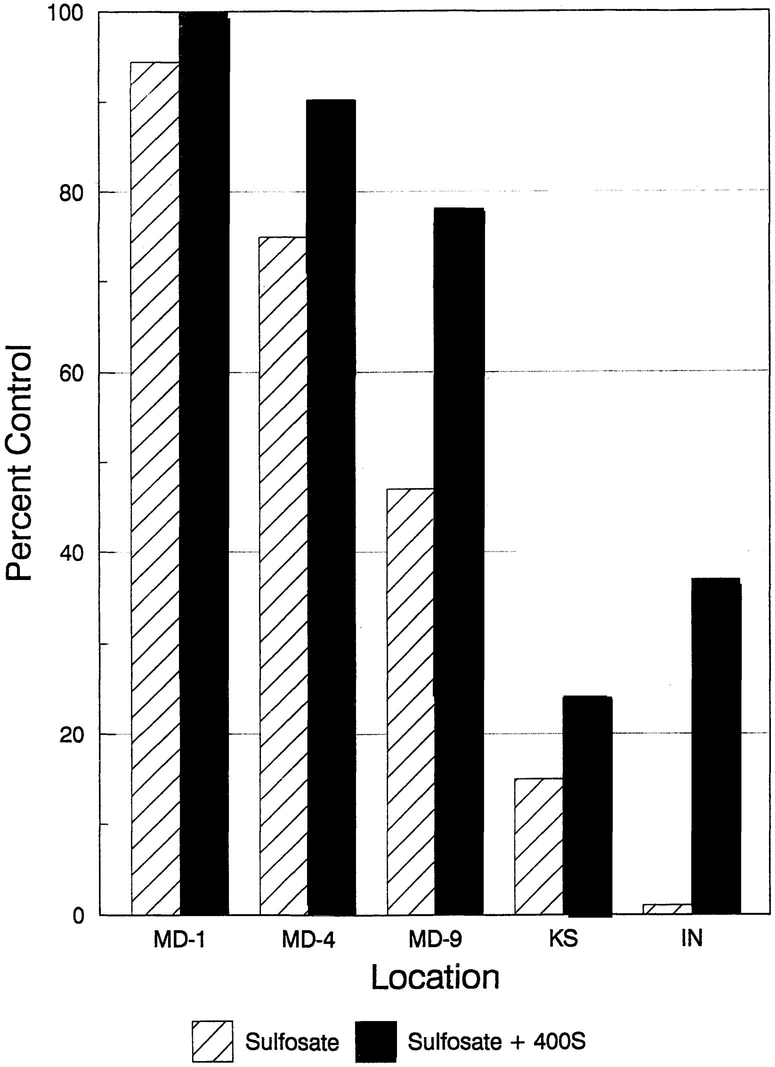


Figure 6. Synergy between sulfosate (0.28 kg ai/ha) and a bacterial preparation (400S) on barnyardgrass at three field locations.

Literature Cited

1. Holliday, M.J.; Keen, N.T. *Phytopathology* 1981, *71*, 881.
2. Quimby, P.C. *Proc. South Weed Sci. Soc.* 1985, *38*, 365-371.
3. Keen, N.T.; Holliday, M.J.; Yoshikawa, M. *Phytopathology* 1982, *72*, 1467-1470.
4. Sharon, A.; Amsellem, Z; Gressel, J. *Plant Physiol.* 1992, *98*, 654-659.
5. Wymore, L.A.; Watson, A.K.; Gotlieb, A.R. *Weed Sci.* 1987, 35, 377-383.
6. Hodgson, R.H.; Snyder, R.H. *Weed Sci.* 1989, *37*, 484-489.
7. Wymore, L.A.; Watson, A.K.; *Weed Sci.* 1989, 37, 498-483.
8. Hodgson, R.H.; Wymore, L.A.; Watson, A.K.; Snyder, R.H.; Collette, A. *Weed Technol.* 1988, 2: 473-480.
9. Caulder, J.D.; Stowell, L. U.S. Patent #4,766,873, 1988, 18PP.
10. Rahe, J.E.; Levesque, C.A.; Johal, G.S. In *Microbes and Microbial Products as Herbicides*, edited by R.E. Hoagland. ACS Symposium Series No. 439; American Chemical Society: Washington, DC 1990, PP. 260-275.
11. Amsellem, Z.; Sharon, A.; Gressel, J.; Quimby, P.C., Jr. *Phytopathology* 1990, *80*, 925-929.
12. Kennedy, A.C.; Elliott, L.F.; Young, F.L.; Douglas, C.L. *Soil Sci. Soc. Am. J.* *55*, 722-727.
13. Kremer, R.J. *Microbial Ecology* 1987, *14*, 29-37.
14. Kremer, R.J.; Begonia, M-F.T; Stanley, L; Lanham, E.T. 1990. *Appl. Eviron. Microbiol.* *56*, 1649-1655.
15. Harris, P.A.; Stahlman, P.W. *Weed Sci. Soc. Amer. Abstracts* 1992, *32*, 50.
16. Baragona, J.S.; White, J.C. *Weed Sci. Soc. Amer. Abstracts* 1992, *32*, 50.

RECEIVED September 14, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch007

Chapter 8

Altering the Host Range of Mycoherbicides by Genetic Manipulation

David C. Sands and R. Vincent Miller

Department of Plant Pathology, Montana State University, Bozeman, MT 59717

New strategies for the development of biological control agents of weeds with plant pathogens are discussed. The basic premise is based on using highly virulent broad-host range pathogens that lack the host-specificity currently thought necessary for bioherbicides. These pathogens could be rendered safe by conferring biological confinement via auxotrophy or reduced survival ability. Additional discussion is on possible methods for genetically constructing host-specificity based on root exudates that are unique to a target host similar to a "Zip code", or using the host itself as a source of self destructive genes, which we refer to as the "Pogo theory" from a quote by the comic strip character, "We have found the enemy and it is us".

The most widespread and important methods of weed control currently used are tillage and chemical herbicides (*1*). The use of herbicides with documented adverse affects on groundwater quality, mammalian toxicity, and human health have led to increased interests in alternatives such as the use of biological control. Despite a few successes though, biological control as it is now practiced cannot be expected to completely replace the use of chemical herbicides. Present biocontrol agents, developed with either the classical or mycoherbicide approach (*2*,*3*), must be host-specific for reasons of environmental safety and liability. And there simply may not be ample prospective host-specific biocontrol agents for each problem weed. Further, economic constraints preclude the use of host-specific agents for many sites with multiple weed species. Thus, the justifications to attempt to shift from chemical herbicides to biological control are neither entirely

0097–6156/93/0524–0101$06.00/0

clear nor convincing. Probably the best justification for biocontrol involves the potential marketing of "pesticide free" crops, but production costs would be higher, at least until better biocontrol agents are available for control of insects, weeds, and fungi. A market may become established if sufficient numbers of customers desire such pesticide-free products.

Biological Containment Systems

It seems likely that, as in the chemical herbicide industry, one or several paradigm shifts will be necessary before any advances are made. The proposed paradigm shift is away from the classical approach of screening only host-specific exotic or native pests to one emphasizing genetic manipulation and an extensive knowledge of physiology and biochemistry of host/ parasite interactions. Our basic proposal is that contained broad-host range pathogens might better compete with broad spectrum herbicides by virtue of their use against multiple target weeds, reduced residue risk, reduced liability, repeated sales requirements, and possibly "perceived" aspects of safety.

Broad-Host Range Plant Pathogens

Broad-host range pathogens, well known by plant pathologists, include *Sclerotinia sclerotiorum*, *Pythium ultima*, *Sclerotium rolfsii*, *Phymatotrichum omnivorum*, and *Pseudomonas solanacearum* (*4*,*5*). Often soil-borne, these pathogens are capable of surviving in the soil after killing one plant until they attack another susceptible plant of the same or different species. Their mode of action involves root or crown invasion, vascular plugging and wilting, and production of considerable biomass for survival (*4*,*5*). The genetically manipulatable aspects of these pathogens are their host range, sexual structures, survival, and their ability to synthesize most if not all vitamins, amino acids, fatty acids, purines and pyrimidines. As with other plant pathogens, the mechanisms involved in host range restriction are unknown.

Sclerotinia sclerotiorum Model System

We have developed a series of mutant strains of *Sclerotinia sclerotiorum* (*6*,*7*). This pathogen normally attacks over 300 genera of plants including the over 30 species of weeds (Table I) (*8*). The objective of this research was to obtain mutants of this broad-host range fungus that either have a reduced host range or are in some other way useful for genetic manipulation. This fungus serves as a model system to demonstrate that broad-host range pathogens in general can be made more useful as biological control agents. We obtained a group of auxotrophic *S.*

Table I. Common Weed-Hosts of *Sclerotinia sclerotiorum*

Scientific Name	Common Name
Medicago lupulina L.	Black Medic
Brassica nigra (L.) W.Koch *B. rapa* L.	Black Mustard Field Mustard
Orobanche sp. L.	Broomrape
Cirsium arvense L. (Scop.) *C. vulgare* (Savi) Ten.	Canada Thistle Bull Thistle
Stellaria media (L.) Vill.	Common Chickweed
Xanthium strumarium L.	Cocklebur
Portulaca oleracea L.	Common Purslane
Sonchus oleraceus L.	Common Sowthistle
Taraxacum officinale Wigg.	Dandelion
Solidago sp. L.	Goldenrods
Senecio vulgaris L.	Common Groundsel
Cannabis sativa L.	Hemp
Centaurea maculosa L. *C. repens* L. *C. solstitialis* L.	Spotted Knapweed Russian Knapweed Yellowstar Thistle
Chenopodium album L.	Lambs-quarters
Lupinus sp. L.	Lupines
Urtica dioica L.	Stinging Nettle
Solanum nigrum L.	Common Nightshade
Chrysanthemum leucanthemum L.	Oxeye Daisy
Thlaspi arvense L.	Field Pennycress
Amaranthus retroflexus L.	Redroot Pigweed
Conium maculatum L.	Poison Hemlock
Ambrosia artemisiifolia L.	Common Ragweed
Capsella bursa-pastoris L. Medik	Shepherd's Purse
Galinsoga parviflora Cav.	Small-flowered Galinosoga
Euphorbia sp.	Spurges
Rumex crispus L.	Curly Dock

sclerotiorum mutants unable to grow without certain nutritional supplements and a series of strains with various degrees of virulence that were unable to make the survival/sexual structures called sclerotia (Table II). Results obtained from field sites established that some of these mutants are still virulent, though less so as compared to the wild-type fungus (Figure 1).

Table II. Current Mutants of *Sclerotinia sclerotiorum*

Mutant	Phenotype	Parental Origin
A1-PYR	Cytosine required for growth	KA2
A2-CYS	Methionine or cysteine required for growth	84.1B
A3-PYR	Cytosine required for growth	A-169
A4-ARG	Arginine required for growth	A-169
A5-LYS	Lysine required for growth	A-169
A6-ARG	Arginine required for growth	A-169
A8-LEU	Leucine required for growth	
A9-ISO/VAL	Isoleucine and valine required for normal growth	A-169
A10-ADE	Adenosine required for growth	CM813-2
A11-CHOL	Choline required for growth	CM813-2
A13-LYS	Lysine required for growth	CM813-2
A14-ISO/VAL	Isoleucine and valine required for normal growth	CM813-2
SL-1	Sclerotialess, low virulence	84.1B
SL-5	Sclerotialess, avirulent	A-9
SL-7	Sclerotialess, moderate virulence	A-169

In the field trial in Figure 1, the fungus was grown in shake cultures and homogenized mycelium used to inoculate sterile canola seed imbibed with appropriate nutrient solutions. The infested canola was applied at 500, 1000 and 2000 lb/ac. *Albeit* high rates of application are used in this field test, the results dramatically illustrate the potential of utilizing genetically delimited, but virulent strains of broad-host range pathogens in biological control. Preliminary laboratory and greenhouse tests

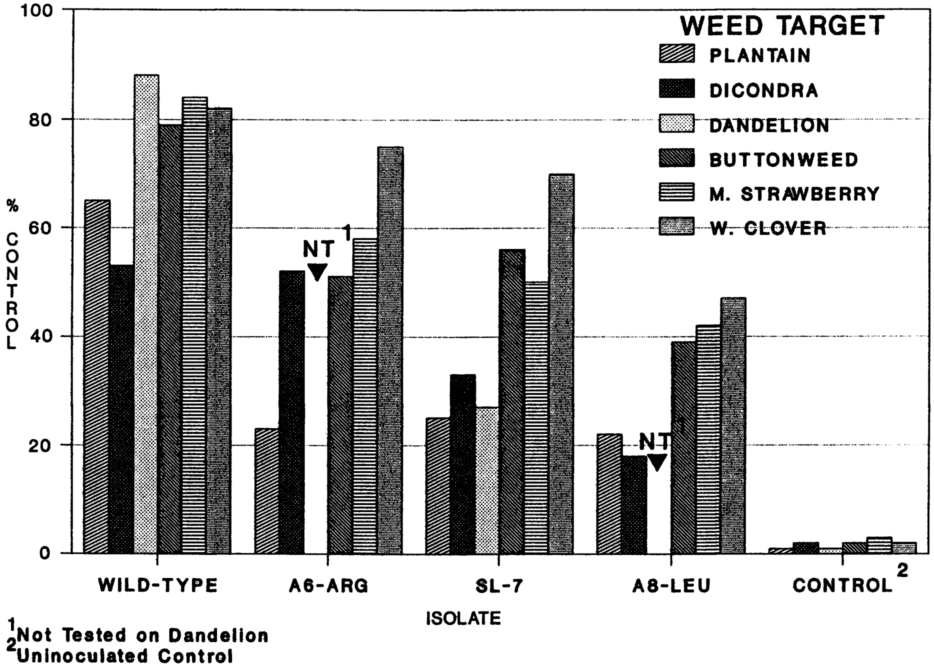


Figure 1. Efficacy of *S. sclerotiorum* mutants compared to the wild-type strain on six turf weeds in field trials conducted in Mississippi. Treatments consisted of 1000 lb/acre fungus infested canola seed imbibed with the one percent tryptone, 10 mM sucrose, and for the auxotrophs, 50 arginine or leucine. Mean of three replications of five foot square plots analyzed after 21 days.

indicate that formulations can significantly reduce necessary application rates. Further, it is our contention that these rates are tolerable as spot treatments in certain home and garden markets.

Strain Improvement via Mating

Auxotrophs of different parent strains might be useful for several other reasons. For example, they could be crossed (lys$^-$ × arg$^-$) to search for genetic recombinants (arg$^+$ and lys$^+$) that might have different useful traits. Secondly, auxotrophs can be tested for virulence in the presence or absence of the appropriate nutrient. One example would be the cytosine minus strains that require external cytosine for infection (*6*). Thirdly, auxotrophs can be used to facilitate genetic modification. The cytosine plus gene can be added by transformation to a cytosine minus strain but on a different promoter. If the promoter only activates transcription in the presence of certain plants, then the previously broad-host range pathogen would be rendered host-specific.

Genetically Constructed Host-Specificity

The key signals that could be used to activate modified pathogens would probably be compounds that are unique to the target host. An example of a target specific compound might be the alkaloid cnicin found in and on knapweed leaves (*9*). This compound might serve as a specific inducer of prototropy in a genetically modified pathogen. For instance, without cnicin the pathogen would require cytosine whereas in the presence of cnicin cytosine would be synthesized.

Natural chemical recognition systems may be available from pathogens, epiphytes, insects, nematodes, or rhizosphere microflora associated with the target host, or even from the host itself. For instance, cnicin utilizing microflora may be obtained from organisms isolated from spotted knapweed itself, soil-borne microorganisms, or from the intestinal flora of insects or nematodes that feed on this plant. These promotor or repressor systems can be cloned upstream to the gene restoring prototropy and then inserted into the pathogen via transformation.

A Zip Code System for Auxotrophy

The root exudates of plants have been well characterized and generally consist primarily of certain amino acids and sugars (*10*). Pathologists have found little relationship between root exudate content and host/parasite specificity (*10*). However, as early as the mid 1950's, Garber (*11,12*) demonstrated a relationship between auxotrophic mutants and cultivar-level host selectivity with *Erwinia aroideae*, precedence for a nutritionally-based system for conferring host-specificity. Other factors that have been implicated in host-specificity include lectin binding, pathogen-specific and non-pathogen specific toxins, spore agglutinating factors, and non-specific defense mechanisms (*13*). We can determine which free amino acids are in high amounts in the subsoil crown of the target plant and, as might be expected, certain amino acids are not common in all crowns of all plant species. A multiple auxotroph could then be designed to specifically attack a certain plant based on its free amino acid profile. An example might be *Centaurea maculosa*, the spotted knapweed which has high amounts of free asparagine, alanine, aspartate, proline, and threonine in its crown (Table III). Proline is highly variable in plants particularly in response to various stresses such as senescence or drought. But an auxotroph requiring the other four amino acids might be able to infect the crown of this plant whereas other non-target hosts might not provide sufficient levels of these essential amino acids. Such "zip coding" is an artificial system that may enable development of host-specificity via auxotrophs.

A General Strategy for Finding Useful Genes to Enhance Specificity or Virulence

Biocontrol agents are not always as specific or virulent (lethal) as required. One potential source of genes to enhance biocontrol agents is from the target host itself. The underlying presumption is that most multicellular organisms contain numerous intermediary metabolites, hormones, and enzymes, that if overproduced or produced at the wrong time would be lethal. We refer to this general strategy as the Pogo Strategy after the Walt Kelly cartoon character who said, "We have found the enemy and it is us." Examples might include molting hormones in nematodes and insects, insect egg hatching factors, seed germination inhibitors or simulators, plant growth hormones, alkaloids, or plant defenses such as phytoalexin production (*14,15,16,17,18*). The genetic approach to this strategy would involve using cDNA in a expression vector and assaying the cloned gene products back on the host. After finding active compounds, these could be used directly or their appropriate genes could be cloned into an appropriate pathogen. Notably, many of the final products listed are multigenic in nature but are often under regulatory systems that may be coded by a single gene and thus accessible to manipulation.

Conclusions

We expect to see several shifts in strategy as biological control becomes less a classical hunting and gathering operation and more involved with genetic manipulation. As ways to make broad-host range pathogens safer are devised, we may see the use of biologicals analogous to that of broad spectrum chemical herbicides.

Table III. Amino Acid Profile of Spotted Knapweed

Amino Acid	μmol/ml[1]
Alanine	0.248
Arginine	0.652
Aspartic Acid	0.523
Cystine	0.039
Glutamic Acid	0.340
Glycine	0.030
Histidine	0.150
Isoleucine	0.092
Leucine	0.041
Lysine	0.056
Methionine	Not Tested
Phenylalanine	0.024
Proline	1.520
Serine	0.342
Threonine	0.327
Tryptophan	0.061
Tyrosine	0.042
Valine	0.214
Asparagine	6.870
Glutamine	1.270
α-Aminobutyric Acid	0.033
Ethanolamine	0.073
Sarcosine	0.047

[1] μmol/ml of an ethanolic extract of crowns.

Literature Cited

1. Anderson, W. P. *Weed Science: Principles, Second Edition*; West Publishing Company, St. Paul, Minnesota, **1989**, pp. 63-117.
2. Watson, A. K. In *Microbial Control of Weeds*, TeBeest, D. O., Ed.; Chapman and Hall, New York, **1991**, pp. 3-23.
3. Charudattan, R. In *Microbial Control of Weeds*, TeBeest, D. O., Ed.; Chapman and Hall, New York, **1991**, pp.24-57.
4. Agrios, G. N. *Plant Pathology, Third Edition*; Academic Press, Inc., San Diego, California; **1988**, pp. 293-313, 430-435, 487-496, 547-549.
5. Lockwood, J. L. *Annu. Rev. Phytopathol.,* **1988**, *26,*93-121.
6. Miller, R. V., Ford, E. J., Zidack, N. J., and Sands, D. C. *J. Gen. Microbiol.* **1989**, *135,*2085-2091.
7. Miller, R. V.; Ford, E. J.; Sands, D. C. *Can. J. Microbiol.* **1989**, *35,*517-520.
8. Farr, D. F.; Bills, G. F.; Chamuris, G. P.; Rossman, A. Y. *Fungi on Plants and Plant Products in the United States*; American Phytopathological Society, St. Paul, Minnesota, **1989**, pp. 941.
9. Locken, L. J.; Kelsey, R. G. *Biochem. System. Ecol.* **1987**, *15,*313-320.
10. Schroth, M. N.; Hildebrand, D. C. *Annu. Rev. Phytopathol.*. **1964**, *2,*101-132.
11. Garber, E. D. *Proc. Nat. Acad. Sci. U.S.* **1954**, *40,*1112-1118.
12. Garber, E. D. *Amer. Natural.* **1956**, *90,*183-194.
13. Daly, J. M. *Annu. Rev. Phytopathol.* **1984**, *22,*273-307.
14. Riddle, D. L.; Georgi, L. L. *Annu. Rev. Phytopathol.* **1990**, *28,*247-269.
15. Mayer, A. M.; Poljakoff-Mayber, A. *The Germination of Seeds, Second Edition*; Pergamon Press, Oxford, England, **1977**, pp. 126-151.
16. Evans, M. L. In *Encyclopedia of Plant Physiology, New Series, Vol. 10, Regulation of Development,* **1980**, pp. 23-79.
17. Derlinger, D. L. In *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*; Kerkut, G. A.; Gilbert, L. I. Eds.; Pergamon Press, Oxford, England, **1985**, *8,*353-412.
18. Truman, J. W. In *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*; Kerkut, G. A.; Gilbert, L. I. Eds.; Pergamon Press, Oxford, England, **1985**, *8,*413-440.

RECEIVED November 23, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

Chapter 9

Natural Phytotoxins as Herbicides

Stephen O. Duke[1] and John Lydon[2]

[1]Southern Weed Science Laboratory, Agricultural Research Service,
U.S. Department of Agriculture, P.O. Box 350, Stoneville, MS 38776
[2]Weed Science Laboratory, Beltsville Agricultural Research Center–West,
Agricultural Research Service, U.S. Department of Agriculture,
Beltsville, MD 20705

Natural products of plants and microbes offer a vast array of secondary compounds with biological activity, including phytotoxicity. Many of these compounds have the potential to be used directly as herbicides or as structural leads for new synthetic herbicides. Although natural compounds have made a large impact in the insecticide area, relatively few successes have been obtained with these compounds as herbicides. The most notable success is that of glufosinate. Use of natural products in a herbicide discovery strategy has been hindered by several problems. The number of options that must be considered in discovery and development of a natural product as a herbicide is larger than for a synthetic herbicide. Furthermore, the molecular complexity, limited environmental stability, and low herbicidal activity of many phytotoxic natural products are discouraging. Rediscovery of known natural phytotoxins can be time-consuming and expensive. However, advances in chemistry and biotechnology are increasing the speed and ease with which humankind can discover and develop natural products as herbicides, while diminishing returns are being experienced with conventional herbicide discovery efforts based on "synthesize and screen" strategies.

No one knows when humans first utilized the biological activity of certain natural materials against pests. There is little doubt, however, that natural products have been used for pest control for thousands of years. Within the past century, we have gained the capacity to isolate, identify, and exploit active pesticidal compounds from natural sources. This capacity has had a major impact in the discovery and development of chemicals for insect control. However, there has been relatively little success with natural compounds for weed control. This review will document successes, near successes, and the potential for future success in developing herbicides from natural compounds.

The topic of natural products as herbicides has been reviewed previously in its entirety (*1, 2*) and as part of more extensive reviews on natural products as pesticides (*3*). Portions of this subject have been covered in various reviews

**This chapter not subject to U.S. copyright
Published 1993 American Chemical Society**

on the pesticidal properties of particular natural product families (*4, 5*) and natural products as herbicides or pesticides from particular sources, such as plants (*6-8*) or microbes (*9-20*). This review will be a critical review that will deal only with the most important developments, the issues, and the outlook for development of herbicides from natural sources.

Need and Rationale

The need for new herbicides will continue into the foreseeable future. Approximately two-thirds, by volume, of the pesticides used in agricultural production are herbicides. Thus, the potential for undesirable environmental contamination from herbicides is relatively great. There is a growing need for more environmentally and toxicologically safe herbicides that are equally or more efficaceous and selective than currently available herbicides. Increased emphasis on reduced-tillage and no-tillage agriculture will, in some cases, make adequate control of weeds more dependent on herbicides. The accelerating incidence of herbicide resistance is creating a need for new herbicides with previously unexploited mechanisms of action. New herbicides will be required to solve the dilemma of the continued need for herbicides while older herbicides are removed from the market for toxicological, environmental, or economic reasons. Compounds from natural sources may offer solutions to some of these needs.

The number of synthetic compounds screened to discover a commercially useful herbicide has increased dramatically due to diminishing returns with this method and more stringent requirements for new herbicides. Furthermore, biorational design strategies, based on targeting specific molecular targets have so far been unsuccessful in discovery of commercially viable herbicides. Therefore, new discovery strategies, such as using natural products as herbicides or leads for new herbicides, must be considered. Natural product-based herbicides may have desirable characteristics such as shorter environmental half-lives than synthetic herbicides that are predominantly halogenated hydrocarbons with relatively long environmental half-lives. Synthetic halogenated hydrocarbons are generally perceived to have more suspect toxicological properties than most natural compounds. Natural compounds are often found to have unique mechanisms of action, unrelated to known mechanisms of commercial herbicides (*1, 9, 15*). Thus, natural compounds have increasingly become a focus of those interested in discovery of herbicides (*1-20*).

There is little interest within the herbicide industry in utilizing crude extracts of organisms as herbicides, even though there are many reports of crude plant extracts with herbicidal activity (*e.g., 21*). The toxicological, quality control, economic, and patent obstacles to development of crude extracts as herbicides are severe. However, there is considerable interest in the utilization of cover crops to generate phytotoxic plant residues in soil to inhibit weed establishment and growth. This short review will discuss only structurally identified natural compounds that have been utilized or considered for weed control.

Plant-Derived Compounds with Herbicidal Potential

There are many reports of the production of phytotoxic symptoms by specific secondary compounds isolated from plants (*6, 7, 22-24*). These compounds with biological activity are often termed allelochemicals. A role of plant-produced compounds has been clearly established in plant-plant interactions in relatively few cases. Although most of the simple, ubiquitous phenolic acids and flavonoids reported to be allelochemicals are weakly phytotoxic, they are ineffective

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

phytotoxins in soil and have little selectivity. For example, ferulic acid, a phenolic acid produced by all plants, has been called an allelochemical (*25*). However, it is only active at millimolar levels in petri dish assays (*e.g., 26*) and, in the field, its activity is decreased significantly due to strong binding to soil and to rapid microbial degradation (*27*). Several of these phenolic compounds (*e.g.*, salicylic acid and *p*-hydroxybenzoic acid), at very high rates (56 to 112 kg/ha), are effective against weeds and are relatively non-selective (*e.g., 28*).

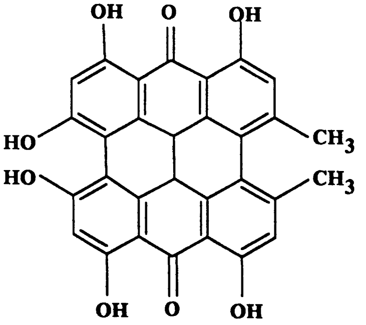
However, synthetic modification of these compounds can increase their efficacy and selectivity. For example, the halogenated benzoic acid herbicides (*e.g.*, dicamba, chloramben, and picloram), are derived from benzoic acid, a phenolic plant product. Some phenolic compounds, such as the coumarins, are much more phytotoxic than others (*e.g., 26*). Synthetic derivatives of coumarins have been reported in patent applications to be good herbicides. Other aromatic compounds, such as 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA, Figure 1), are as active in reducing plant growth as many herbicides (*29, 30*). Phenolic derivatives such as the dihydroquinone sorgoleone (Figure 1), produced by *Sorghum bicolor*, are extremely phytotoxic in hydroponic culture (*31*). Various phenolic quinones such as juglone (Figure 1) and lawsone are among the more phytotoxic phenolic compounds (*32, 33*).

Because natural levels of these plant-produced phenolic compounds are unlikely to significantly affect plant growth under most conditions, some investigators have attempted to prove that synergism between these weak phytotoxins produces a signifcantly phytotoxic combination. Synergistic interactions in causing phytotoxicity have been reported in poorly planned studies (*e.g., 34, 35*). However, more careful studies have found additive or antagonistic effects (*26, 36, 37*).

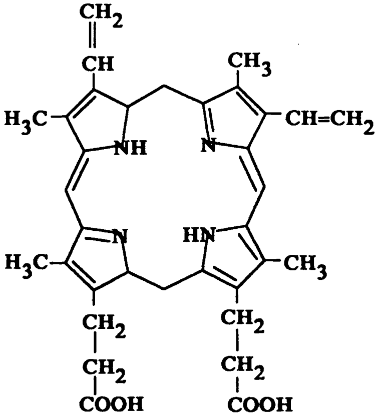
Some of the most convincing reports of allelochemical involvement in plant competition involve terpenoids (*e.g., 38*). For example, the monoterpene 1,8-cineole (Figure 1) is strongly phytotoxic. A 1,8-cineole derivative, cinmethylin (Figure 1), has been seriously considered for herbicide development (*39, 40*). A mixture of about 200 different compounds produced by polychlorinating camphene (Figure 1) was used as both an insecticide and a herbicide (*41*). However, this pesticide was discontinued because of toxicological risks. The phytotoxic compound(s) responsible for its herbicidal activity was never identified, and it is possible that the phytotoxic component(s) may have posed no health risk. The sesquiterpene lactone artemisinin (Figure 1), an antimalarial compound from annual wormwood (*Artemisia annua* L.), is very phytotoxic (*5, 42-45*). However, its complex structure alone precludes it from consideration as a commercial herbicide because of difficulties in synthesis. Other highly phytotoxic plant-derived terpenoids have been described (*e.g., 4, 5, 46, 47*).

Higher plants produce many photodynamic compounds, such as α-terthienyl and hypericin (Figure 1) (*48*). Although these compounds are strongly phytotoxic, they are unlikely to be developed as herbicides because, in the presence of light, they are toxic to all organisms. A compound applied to plants that would make them generate toxic levels of endogenous photodynamic compounds would be more toxicologically acceptable. Plants can be caused to generate phytotoxic levels of photodynamic porphyrins by treatment with a combination of two non-phototdynamic compounds, the natural porphyrin precursor δ-aminolevulinic acid (ALA) and 2,2'-dipyridyl, a synthetic compound (*49-51*). Considerable effort has gone into development of ALA as a herbicide. However, it has recently been found that several classes of commercial herbicides kill plants by causing the accumulation of toxic levels of the photodynamic porphyrin intermediate, protoporphyrin IX (Figure

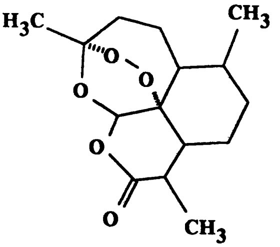
Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009


OH
O
OH
HO
HO
CH3
CH3
OH
O
OH

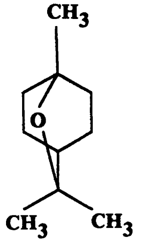
HYPERICIN


CH2
CH
CH3
H3C
CH=CH2
NH
N
N
HN
H3C
CH3
CH2
CH2
COOH
CH2
CH2
COOH

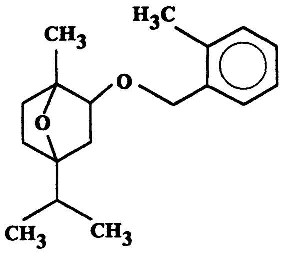
PROTOPORPHYRIN IX


H3C
CH3
O
O
O
O
O
CH3

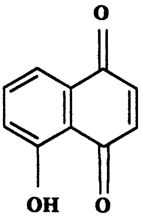
ARTEMISININ


CH3
O
CH3
CH3

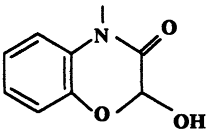
1,8-CINEOLE


H3C
CH3
O
O
CH3
CH3

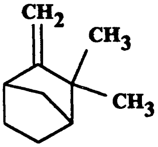
CINMETHYLIN



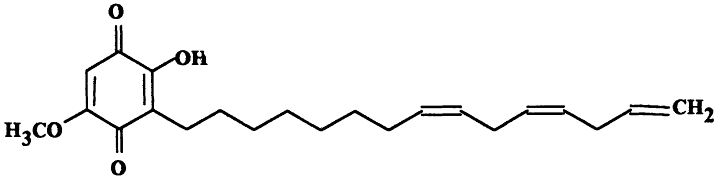
JUGLONE



DIBOA



CAMPHENE


O
OH
H3CO
O
CH2

SORGOLEONE

Figure 1. Structures of plant-derived phytotoxins mentioned in the text.

In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.

1) (*51-54*). This topic is dealt with in detail in another chapter in this volume. Even if protoporphyrin IX were inexpensive, it is not effective as a herbicide when applied exogenously (*55*).

Certain alkaloid (*e.g.*, colchicine and vinblastine) and terpenoid (*e.g.*, taxol) plant products that stop growth of plants by interference with mitosis (*56, 57*) have modes of action identical to certain synthetic herbicides. Little attention has been given to their potential as herbicides, due, in part, to difficulty in synthesizing these complex molecules and the low yield from producing plants. Some of these compounds are extremely expensive as pharmaceuticals, which have the advantage of being sold at a much higher price per unit mass than can pesticides (*e.g.*, taxol). Simpler alkaloids such as caffeine can, in some cases, act as selective herbicides at extremely high application rates (*28, 58*).

Unaltered, plant-produced phytotoxins are generally weakly active compared to commercial herbicides. Most known allelochemicals would have to be applied at rates of more than 10 kg/ha to achieve significant weed control, whereas, many recently introduced herbicides can achieve the same level of control at levels three orders of magnitude smaller. When comparing two herbicides, application rate does not always correlate directly with enviromental risk or cost. However, there is little interest in the herbicide industry in compounds that are only effective at rates of more than a kilogram per hectare.

That plants do not produce large numbers of highly phytotoxic compounds is not unexpected, because production of such compounds would lead to strong autotoxicity unless the producing plant developed metabolic or physical mechanisms to cope with its own phytotoxins. Some of the more potent allelochemicals are toxic to the producing species and this autotoxcity has been implicated in vegetation shifts during ecosystem succession.

The compounds from plants that might be most profitably used as leads for new herbicides are enzyme substrates and cofactors. Substrate and cofactor analogues can be potent inhibitors, resulting in strong herbicidal activity. For example several herbicidal chemical classes are competitive inhibitors of protoporphyrinogen oxidase (*59*) by virtue of their structural and electronic similarity to one-half of the protoporphyrinogen molecule (*60*). This strategy, however, is more properly categorized as biorational design rather than that of using natural products as herbicides or leads for herbicides.

Microbial conversion of relatively non-phytotoxic plant-derived compounds in the soil to highly phytotoxic derivatives has also been documented (*e.g., 8*). However, these compounds must be considered microbial products rather than those from plants.

The biological activity of plant-produced secondary compounds is generally much higher against pathogens and insects. Chemical defence is probably the best method that plants have for combating insects or pathogens. However, allelopathy does not appear to be a prevalent strategy in plant-plant interference. Direct competition for resources is the more common plant-plant interaction. Perhaps because of the relative paucity of potent phytotoxins, the pesticide industry has been much more successful in exploiting plant-derived natural products as insecticides or as templates for insecticides (*e.g.,* pyrethroids) than as herbicides. The potential of microbial products as a source of new herbicides is generally perceived to be much greater than that of plants.

Microbial Compounds with Herbicidal Potential

Microbial compounds have been more successfully exploited either as herbicides or leads for new herbicides than plant-derived compounds (*e.g., 1, 2, 10, 15*).

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

Several strategies may be employed in utilizing microbial products as new herbicides. Either non-pathogenic microbes such as soil microflora (*e.g.*, many of the actinomycetes) or plant pathogens can be utilized as sources of phytotoxins. Non-pathogenic microbes have been the source of natural compounds most commonly utilized by industry in pesticide discovery. They are relatively easily cultured compared to pathogens, and they produce a multitude of bioactive products.

A major problem with these organisms is inadvertant rediscovery of known phytotoxins, such as cycloheximide or gabaculine, after expensive and time-consuming studies (*e.g., 20, 61*). Quality control in bioassays and carefully documented profiles of known compounds in those bioassays can be used to screen out probable known compounds. In one company's herbicide discovery program, only 28% of all identified phytotoxic compounds from microbes were new structures and another 16% were known structures, previously not known to be phytotoxic (*20*). Many of these compounds were amenable to analogue synthesis.

Phytotoxins from microbes offer excellent opportunities for identifying potential molecular target sites for herbicide design. There is very little overlap between the known molecular sites of action of commercial synthetic herbicides and those of microbial phytotoxins (*1, 2, 9, 15*). A great deal is known about the mechanisms of action of microbial phytotoxins that could be exploited by the herbicide industry (*62-64*).

The only significant commercial successes of herbicides either from or based on microbial products have been from non-pathogens. Bialaphos (Figure 2), a tripeptide with herbicidal activity was first isolated from cultures of *Streptomyces viridochromognes* and was later found to be also produced by *Streptomyces hygroscopicus* (*65-67*). Bialaphos is composed of a glutamic acid analog called phosphinothricin and two alanine residues. In plants and bacteria, bialaphos is metabolized by hydrolysis of the alanyl groups to produce phosphinothricin, which inhibits glutamine synthetase from bacteria and plants, thereby disrupting amino acid synthesis (*65, 68-72*). This metabolic site is not targeted by any herbicides developed by other strategies. Bialaphos and synthetic phosphinothricin (glufosinate, Figure 2), are the active ingredients in commercial, non-selective herbicides.

The herbicidally active tripeptide phosalacine produced by *Kitasatosporia phosalacinea* sp. nov. KA-338 differs from bialaphos in that the terminal amino group is a leucine residue (Figure 2) (*73*). The toxicity of phosalacine was attributed to it being metabolized to phosphinothricin in affected organisms (*74*). Interestingly, glufosinate is similar to one of the most succesful synthetic, non-selective herbicides, glyphosate. Both are phosphonate amino acid analogs that disrupt amino acid biosynthesis and are readily metabolized in the soil (*75-78*).

Several other phytotoxic compounds produced by *Streptomyces* species also appear to be antimetabolites of amino acids. *Cis*-2-amino-1-hydroxycyclobutane-1-acetic acid (CBAA) produced by *Streptomyces rochei* (*79*) is a constituent of a naturally occurring antibiotic dipeptide (*80*). CBAA causes methionine- and cysteine-reversible chlorosis in plants. Oxetin, a CBAA analog, is produced by a *Streptomyces* sp. and inhibits glutamine synthetase (*81*). Vulgamycin (Figure 2), produced by several *Streptomyces* sp., apparently inhibits isoleucine synthesis and isoleucine-requiring processes (*82*). The antibiotic activity of the herbicidally active compound homoalanosine, produced by *Streptomyces galilaeus*, could be reversed by aspartate or glutamate (*83*)

Phytotoxins from non-pathogenic microbes make up a large proportion of the patented herbicides of microbial origins, many of which have unknown mechanisms of action. Examples of these compounds are toyocamycin, anisomycin and several close

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

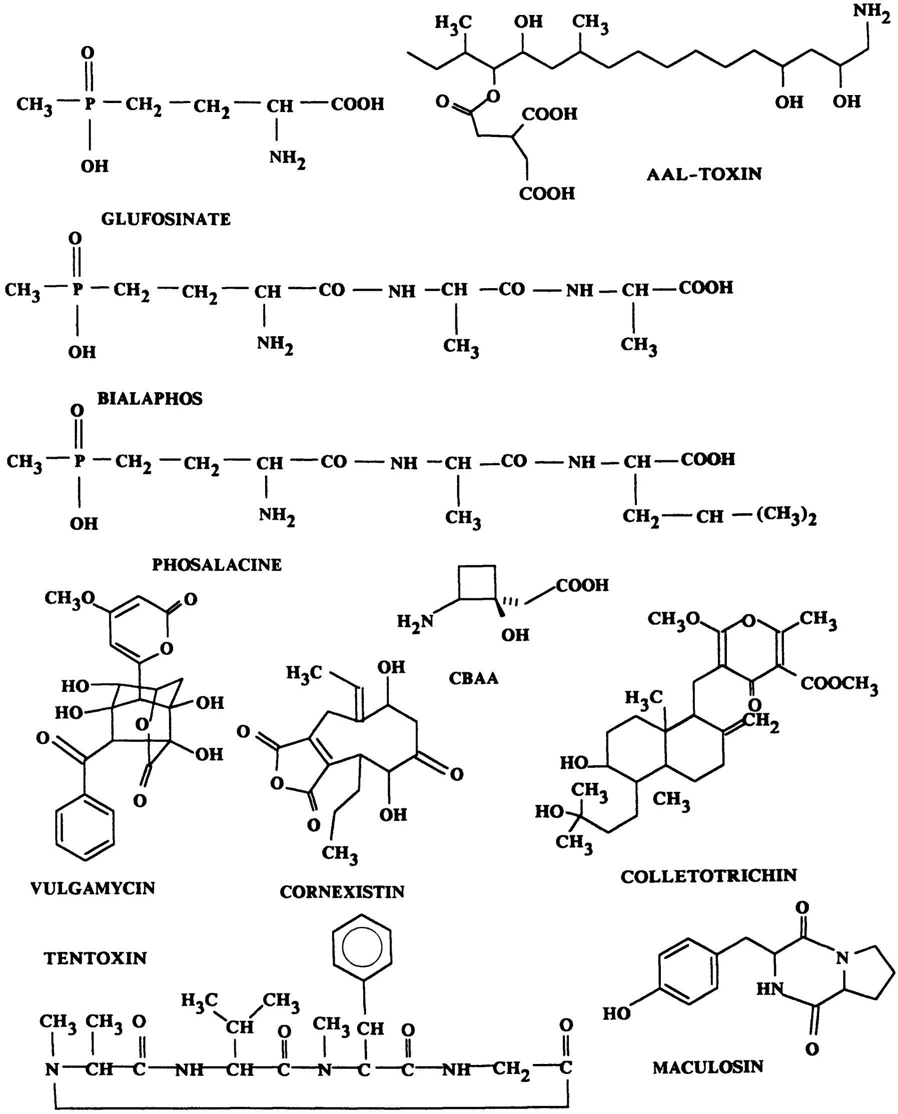

GLUFOSINATE
AAL-TOXIN
BIALAPHOS
PHOSALACINE
CBAA
VULGAMYCIN
CORNEXISTIN
COLLETOTRICHIN
TENTOXIN
MACULOSIN

Figure 2. Structures of microbial phytotoxins mentioned in the text.

analogues, herbimycins, herbicidins, hydantocidin, gelanamycin, nigiricin, and α-methylene-β-alanine (*84-92*). These and many others are discussed in more extensive reviews (*e.g., 1, 10, 19*).

Another example of an interesting phytotoxin from a non-pathogenic microbe is cornexistin (*93*). Cornexistin (Figure 2), a nonadride compound, was isolated from a saprophytic fungus (*Paecilomyces variotii* SANK 21086) found growing on deer dung in Alberta, Canada. It is an effective herbicide for crabgrass, green foxtail, johnsongrass, black nightshade, cocklebur, ragweed, tall morningglory and velvetleaf. However, maize is highly resistent to the compound. Its mechanism of action is unknown, although it is a slow acting phytotoxin that is almost equally effective in light and darkness (T. Amagasa and S. O. Duke, unpublished).

Plant pathogens often produce several phytotoxins as virulence factors (*64*). Thus, the probability of a plant pathogen producing a phytotoxin is good. However, plant pathogens are generally more difficult to culture and to induce to produce toxins in culture than non-pathogens. Some of the compounds are too specific in their selectivity to have any commercial appeal as a herbicide. For these reasons, the herbicide industry has committed relatively few resources to the evaluation of plant pathogens as sources of herbicides. Most of the activity in this area has been the testing of compounds discovered by those outside the pesticide industry. Phytotoxins from plant pathogens as potential herbicides has been the focus of several reviews (*e.g., 17-19*).

Although phytotoxins from pathogens that infect crops are likely to be toxic to some weed species, in most cases, there is no published record on the effect of already discovered phytotoxins on a range of weed species. Little effort has been expended in studying pathogens that infect weeds or the phytotoxins that weed pathogens might produce. All known host-specific phytotoxins except one are produced by pathogens that infect crop species. These compounds are often highly complicated in structure and too selective for herbicidal use. An exception is maculosin (Figure 2), a relatively simple cyclic diketopiperazine analogue of cyclic L-tyrosine-L-proline, is a host-specific phytotoxin from *Alternaria alternata* that appears to affect only spotted knapweed (*Centaurea maculosa*) (*94*). Its mechanism of action is unknown.

Non-host specific toxins are of considerably more interest. An example is phaseolotoxin, produced by *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of halo blight of bean. It has characteristics similar to those of bialaphos (*95-98*). It is a tripeptide that is metabolized in plants or bacteria to an amino acid analog (octicidin) that disrupts amino acid synthesis by inhibition of arginine synthesis (*99-104*). Tabtoxin, a dipeptide protoxin produced by several *P. syringae* pathovars, is hydrolyzed by the producing organism or within the plant host to the phytotoxin tabtoxinine-β-lactam (*105-108*). Tabtoxinine-β-lactam, a phosphinothricin analog, is also an irreversible inhibitor of glutamine synthetase, with herbicidal activity similar to that of bialaphos (*109-111*). There may be no interest in development of this compound as a herbicide because bialaphos and glufosinate already fill this market niche, and tabtoxinine-β-lactam has poor stability.

Another example of a non-host specific phytotoxin from a pathogen is tentoxin (Figure 2), a cyclic tetrapeptide produced by several *Alternaria* species. It causes severe chlorosis in many of the problem weed species associated with soybeans and maize without affecting either crop (*112*). Furthermore, it has two mechanisms of action which may be related; inhibition transport of certain nuclear-coded proteins into plastids (*113*) and inhibition of chloroplast coupling factor ATPase activity (*114*). The synthesis of tentoxin and its

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

production by fermentation are currently too expensive for commercialization. Extensive structure-activity research has not produced a simpler, less expensive compound with similar activity (*115, 116*). However, efforts have been made to understand the genetics of tentoxin biosynthesis so that the cost of its production by fermentation technology can be reduced (*e.g., 117*).

Some phytotoxins from plant pathogens such as the colletotrichins and the fumonisins have relatively rapid effects on membrane integrity. Colletotrichin (Figure 2), a product of several species of *Colletotrichum*, causes rapid leakage of plant cell membranes of several species via an unknown mechanism (*118*). Fumonisin B_1, a non-host specific phytotoxin of *Fusarium moniliforme*, causes rapid loss of plasmalemma and tonoplast integrity, resulting in cellular leakage and eventual cell death to a wide range of weed species (*15, 119*). Its mammalian toxicity is too high (*120*) for serious consideration as a herbicide. However, structure/activity manipulation might result in a good phytotoxin without mammalian phytotoxicity, provided that the mechanism of action of the compound differs between animals and plants. The mechanism of action of fumonisin in animals appears to be inhibition of the synthesis of sphingolipids (*121*), important constituents of brain tissues. Whether this compound exerts its effects on plants by inhibition of phytosphinogolipids is not known. A closely related phytotoxin AAL-toxin (Figure 2) was reported to be a host-selective toxin that exerted its phytotoxicity by inhibition of nucleotide synthesis by inhibition of aspartate carbamoyl-transferase (*122*). However, this phytotoxin is neither host-selective nor does it appear to exert its phytotoxicity by inhibition of nucleotide synthesis (15, 119, 123, 124). In the tomato varieties from which the original AAL-toxin findings were made, fumonisin shows the same effects and resistance patterns (125, T. Tanaka, H. K. Abbas, and S. O. Duke, unpublished results).

Many other non-host specific toxins from plant pathogens have been identified and characterized (*e.g., 126, 127*). One cannot assume that they have all been tested for their potential as herbicides. In discovery efforts based on screening microbial products, compounds discovered to have herbicidal potential are often previously reported compounds (*20*).

Factors Influencing Discovery and Development

There are potentially more steps in the discovery and development of a herbicide based on a natural product than in the discovery and development of a herbicide from a synthesis effort (*6*). Isolation and identification of the compound are required steps in discovery of natural compounds for use as herbicides. Isolation can be a major undertaking if the compound is present in only minute amounts. Until the compound is purified, microbioassays must be relied on for detection of the compound. Many natural products are labile to the extent that extraction procedures may change structure and biological activity. Structure elucidation can be extremely difficult with highly complex compounds. However, improving methods continue to reduce the cost and time associated with these steps.

Many natural products are structurally too complicated to be synthesized economically for agricultural use. For example, these compounds commonly have several chiral centers. Two strategies are available to overcome this problem. Structure-activity studies might result in discovery of a simpler molecule with a commercially viable ratio of synthesis cost to herbicidal activity. An example of a synthesized herbicide that was developed from a more complex microbial product is methoxyphenone which was derived from anisomycin, a *Streptomyces* metabolite (*128*). Alternatively, the molecule might be biosynthetically produced by fermention (*e.g.,* bialaphos) (*129*).

Even if a natural product could be synthesized economically, an analogue rather

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

than the native compound might be more desirable to market. If the phytotoxic nature of the natural phytotoxin or a related natural compound has been reported previously, the patent may be less defensible than that of the synthetic analogue, especially if there is no mention of its source of the synthetic analogue. However, nature may have already optimized the herbicidal structure of compounds which are used primarily as phytotoxins by the producing organism (*e.g.*, phytotoxins of plant pathogens). This view is born out the the lack of success in structure-activity studies with tentoxin (*115, 116, 130*) and phosphinothricin (*131, 132*).

Two factors are peculiar to microbial products. Culture methods (*e.g.*, liquid or solid culture) and culture conditions, both nutritive and environmental, can have a profound influence on production of secondary products by microbes. Thus, two laboratories may screen the same microorganism and obtain entirely different results. To a lesser extent, the same plant can produce different secondary products under different environments (*22*), and different biotypes of the same plant species may produce different secondary products or differ in capacity to produce particular compounds. Chemical or biological inducers can elicit production of high levels of secondary compounds (phytoalexins) which may be present in only trace amounts in unchallenged plants (*133*).

In addition to new compounds and chemical classes, microbial phytotoxins are sources of new sites of action. Considering the relatively few potential sites of action of synthetic herbicides, this aspect of microbial toxins may become more important. There is little overlap between the known sites of action of microbial phytotoxins and commercial herbicides (*1, 9, 15*). Some screening programs for microbial compounds with herbicidal action have been based on the mechanism of action.

Good microbioassays are often required for screening natural products for herbidical activity because of extremely limited amounts of available test compound. These types of assays are not as standardized as those used for more traditional herbicide screens. Examples of such bioassays are *Lemna* sp. grown in microtitre plates, germination and root growth of small-seeded species, and coleoptile growth assays (*1, 11*).

There is no good reason that a natural product should have less regulatory scrutiny than a synthetic compound. Some of the most potent mammalian toxins are natural biosynthetic compounds (*e.g.*, aflatoxins). Thus, although the regulatory requirements for registration of biocontrol agents are relatively lax, it may cost as much to develop a natural compound as a herbicide as it would a synthetic compound because it would fall under the same regulations as a synthetic pesticide.

Summary and Outlook

Natural products offer an amazing array of chemical structures with biological activity, including phytotoxicity. Molecular target sites that have not been exploited by the herbicide industry are often found to be the sites of action of natural phytotoxins. Thus, many of these compounds have potential as herbicides or as templates for new herbicide classes. Nevertheless, only two related currently used commercial herbicides (glufosinate and bialaphos) have been derived from natural compounds. Still, most herbicide manufacturers have some level of herbicide discovery effort associated with natural compounds. Obstacles to success include: structural complexity, resulting in costly structure determination and synthesis; the relative complexity of the discovery strategy of utilizing natural compounds; the very small amounts of compounds usually available for initial bioassays; and time-consuming and costly rediscovery of known compounds. Nevertheless, interest in this strategy continues and is perhaps growing for

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

several reasons. These include: the success of glufosinate and bialaphos; diminishing returns or lack of successes with other discovery strategies; improved extraction and analytical methods; and novel nature of many natural compounds and their mechanisms of action.

Literature Cited

1. Duke, S. O. *Rev. Weed Sci.* **1986**, *2*, 15-44.
2. Duke, S. O.; Lydon, J. *Weed Technol.* **1987**, *2*, 122-128.
3. Rice, E. L. *Pest Control with Nature's Chemicals.* Univ. Okalahoma Press, Norman, OK, 1983, 224 pp.
4. Duke, S. O. In *Handbook of Natural Toxins, Vol. 6, Toxicology of Plant and Fungal Compounds*; Keeler, R. F.; Tu, A. T., Eds.; Marcel Dekker, New York, 991; pp. 269-296.
5. Duke, S. O.; Paul, R. N.; Lee, S. M. *Amer. Chem. Soc. Symp. Ser.* **1988**, *380*, 318-334.
6. Duke, S. O. In *Advances in New Crops,* Janick, J.; Simon, J. E., Eds.; Timber Press, Portland, OR, 1990; pp. 511-517.
7. Lydon, J.; Duke, S. O. In *Herbs, Spices and Medicinal Plants - Recent Advances in Botany, Horticulture, and Pharmacology,* Craker, L. E.; Simon, J. E., Eds; Oryx Press, Phoenix, AZ, 1989, Vol. 4; pp. 1-41.
8. Putnam, A. R. *Weed Technol.* **1988** *2*, 510-518
9. Duke, S.O. In *The Science of Allelopathy,* Putnam, A. R.; Tang, C. S., Eds; John Wiley, New York, 1986; pp. 287-304.
10. Hoagland, R. E. *Amer. Chem. Soc. Symp. Ser.*, **1990**, *439*, 1-52.
11. Cutler, H. G. In *Handbook of Natural Toxins, Vol. 6, Toxicology of Plant and Fungal Compounds*; Keeler, R. F.; Tu, A. T., Eds.; Marcel Dekker, New York, 1991; pp. 411-438.
12. Cutler, H. G. *Weed Technol.* **1988** *2*, 525-532.
13. Fischer, H.-P.; Bellus, D. *Pestic. Sci.* **1983**, *14*, 334-346.
14. Misato, T.; Yamaguchi, I. *Outlook Agric.* **1984**, *13*, 136-139.
15. Duke, S. O.; Abbas, H. K.; Boyette, C. D.; M. Gohbara *Brighton Crop Prot. Conf., Weeds - 1991* **1991**, *1*, 155-164.
16. Poole, N. J.; Chrystal, E. J. T. *Brighton Crop Prot. Conf., Weeds - 1985* **1985** *2*, 591-600.
17. Strobel, G.; Kenfield, D.; Bunkers, G.; Sugawara, F.; Clardy, J. *Experientia* **1991** *47*,819-826.
18. Kenfield, D.; Bunker, G.; Strobel, G. A.; Sugawara, F. *Weed Technol.* **1988** *2*, 519-524.
19. Strobel, G. A.; Kenfield, D.; Bunkers, G. In *Handbook of Natural Toxins, Vol. 6, Toxicology of Plant and Fungal Compounds*; Keeler, R. F.; Tu, A. T., Eds.; Marcel Dekker, New York, 1991; pp. 397-409.
20. Ayer, S. W.; Isaac, B. G.; Krupa, D. M.; Crosby, K. E.; Letendre, L. J.; Stonard, R. J. *Pestic. Sci.* **1989**, *27*, 221-223.
21. Grainage, M.; Ahmed, S. *Handbook of Plants with Pest-Control Properties.* John Wiley and Sons, New York, 1988, 470p.
22. Rice, E. L. *Allelopathy, 2nd Edt.* Academic Press, New York, 1984, 422pp.
23. Putnam, A. R. In *Weed Physiology, Vol. I. Reproduction and Ecophysiology*; Duke, S. O., Ed.; CRC Press, Boca Raton, FL, 1985; pp. 131-155.
24. Newman, E.I. *Pestic. Sci.* **1982** *13*, 575-582.
25. Einhellig, F. A.; Rasmussen, J. A. *J. Chem. Ecol.* **1979** *5*, 815-824.
26. Duke, S. O.; Williams, R. D.; Markhart, A. H. *Ann. Bot.* **1983** *52*, 923-926.
27. Dalton, B. R.; Blum, U.; Weed, S. B. *Soil Biol. Biochem.* **1989** *21*, 1011-1018.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

28. Shettel, N. L.; Balke, N. E. *Weed Sci.* **1983** *31,* 293-298.
29. Barnes, J. P.; Putnam, A. R.; Burke, B. A. In *The Science of Allelopathy*; Putnam, A. R.; Tang, C. S., Eds; Wiley-Interscience, New York, 1986; pp. 271-286.
30. Wolf, R. B.; Spencer, G. F.; Plattner, R. D. *J. Nat. Prod.* **1985** *48*, 59.
31. Einhellig, F. A.; Souza, I. F. *J. Chem. Ecol.* **1992** *18*, 1-11.
32. Shilling, D. G.; Yoshikawa, F. *Amer. Chem. Soc. Symp. Ser.* **1987** *14*, 335-342.
33. Spencer, G. F.; Tjarks, L. W.; England, R. E.; Seest, E. P. *J. Nat. Prod.* **1986** *49*, 530-533.
34. Einhellig, F. A.; Rasmussen, J. A. *J. Chem. Ecol.* **1979** *5*, 815-824.
35. Rasmussen, J. A.; Einhellig, F. A. *Plant Sci. Lett.* **1979** *14*, 69-74.
36. Blum, U.; Dalton, B. R.; Rawlings, J. O. *J. Chem. Ecol.* **1984** *10*, 1169-1191.
37. Blum, U.; Gerig, T. M.; Weed, S. B. *J. Chem. Ecol.* **1989** *15*, 2413-2423.
38. Muller, W. H.; Muller, C. H. *Bull. Torrey Bot. Club* **1964** *91*, 327-330.
39. Grayson, B. T.; Williams, K. S.; Freehauf, P. A.; Pease, R. R.; Ziesel, W. T.; Sereno, R. L.; Reinsfelder, R. E. *Pestic. Sci.* **1987** *21*, 143-153.
40. May, J. W.; Goss, J. R.; Moncorge, J. M.; Murphy, M. W. *Proc. Br. Crop Protect. Conf.* **1985** *12*, 265-270.
41. Sherman, M. E.; Thompson, L.; Wilkinson, R. E. *Weed Sci.* **1983** *31*, 622-627.
42. Duke, S. O.; Vaughn, K. C.; Croom, E. M.; Elsohly, H. N. *Weed Sci.* **1987** *35,* 499-505.
43. DiTomaso, J. M.; Duke, S. O. *Pestic. Biochem. Physiol.* **1991** *39*, 158-167.
44. Chen, P. K.; Leather, G. R. *J. Chem. Ecol.* **1990** *16*, 1867-1876.
45. Chen, P. K.; Polatnick, M.; Leather, G. *J. Agric. Food Chem.* **1991** *39*, 991-994.
46. Yoshida, S.; Asami, T.; Kawano, T.; Yoneyama, K.; Crow, W. D.; Paton, D. M.; Takahashi, N. *Phytochemistry* **1988** *27*, 1943-1946.
47. Goldsby, G.; Burke, J. A. *Phytochemistry* **1987** *26*, 1059-1063.
48. Towers, G. H. N.; Arnason, J. T. *Weed Technol.* **1988** *2*, 545-549.
49. Rebeiz, C. A.; Montazer-Zouhoor, A.; Mayasitch, J. M.; Tripathy, B. C.; Wu, S.-M.; Rebeiz, C. C. *Amer. Chem. Soc. Symp. Ser.* **1988** *339*, 295-328.
50. Rebeiz, C. A., Nandihalli, U. B.; Reddy, K. N. In *Topics in Photosynthesis - Vol. 10. Herbicides,* Baker, N. R.; Percival, M. P., Eds.; Elsevier Science Publishers, Amsterdam, 1991; pp. 173-208.
51. Duke, S. O.; Becerril, J. M.; Matsumoto, H.; Sherman, T. D. *Amer. Chem. Soc. Symp. Ser.* **1991** *449*, 371-386.
52. Duke, S. O.; Becerril, J. M..; Lydon, J.; Matsumoto, H.; T.D. Sherman *Weed Sci.* **1991** *39*, 465-473.
53. Duke, S. O.: Becerril, J. M.; Sherman, T. D.; Lydon, J.; Matsumoto, H. *Pestic. Sci.* **1990** *30*, 367-378.
54. Scalla, R.; Matringe, M.; Camadro, J.-M.; Labbe, P. *Z. Naturforsch.* **1990** *45c*, 503-511.
55. Lydon, J.; Duke, S. O. *Pestic. Biochem. Physiol.* **1988** *31*, 74-83.
56. Vaughan, M. A.; Vaughn, K. C. *Weed Technol.* **1988** *2*, 533-539.
57. Vaughn, K. C.; Vaughan, M. A. *Amer. Chem. Soc. Symp. Ser.* **1988** *380*, 273-293.
58. Rizvi, S. J. H.; Mukerji, D.; Mathur, S. N. *Agric. Biol. Chem.* **1981** *45*, 1255-1256.
59. Camadro, J.-M.; Matringe, M.; Scalla, R.; Labbe, P. *Biochem. J.* **1991** *277*, 17-21.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

60. Nandihalli, U. B.; Duke, M. V.; Duke, S. O. *Pestic. Biochem. Physiol.* - In Press.
61. Heisey, R. M.; Kishra, S. K.; Putnam, A. R.; Miller, J. R.; Whiteneck, C. J.; Keller, J. E.; Huang, J. *Amer. Chem. Soc. Symp. Ser.* **1988** *380*, 65-78.
62. Durbin, R. D. *Experientia* **1991** *47*, 776-783.
63. Ballio, A. *Experientia* **1991** *47*, 784-790.
64. Mitchell, R. E. *Annu. Rev. Phytopathol.* **1984** *22*, 215-245.
65. Bayer, E.; Gugel, K.H.; Hagenmaier, H.; Jessipow, S.; König, W.A.; Zähner, H. *Helv. Chim. Acta* **1972** *55*, 224-239.
66. Kondo, Y; Ogawa, Y; Tsuruoka, T.; Watanabe, H.; Totsukawa, K.; Suzuke, T.; Moriyama, C.; Yoshida, J.; Inouye, S.; Niida, T. *Sci. Rep. Meiji Seika Kaisha* **1973**, *13*, 34-41.
67. Ogawa, Y.; Tsuruoka, T.; Inouye, S.; Niida, T. *Sci. Rep. Meiji Seika Kaisha* **1973**, *13*, 42-48.
68. Leason, M.; Cunliffe, D.; Parkin, D.; Lea, P. J.; Miflin, B. J. *Phytochemistry* **1982** *21*, 855-857.
69. Manderscheid, R.; Wild, A. *J. Plant Physiol.* *123*, 136-142.
70. Tachibana, K.; Watanabe, T.; Sekizawa, Y.; Takematsu, T. *J. Pestic. Sci.* **1986** *11*, 27-31.
71. Ezaki, N.; Amano, S.; Fukushima, K.; Shigeharu, I.; Niida, T. *Sci. Rep. Meiji Seika Kaisha* **1973** *13*, 60-63
72. Ridley, S. M.; McNally, S. F. *Plant Sci.* **39**, 31-36.
73. Omura, S.; Hinotozawa, T.; Imamura, N.; Murata, M. *J. Antibiot.* **1984** *37*, 939-940.
74. Omura, S.; Murata, M.; Hanaki, H.; Hinotozawa, T.; Oiwa, R.; Tnanka, H.; *J. Antibiot.* **1984** *37*, 829-835.
75. Smith, A. E. *J. Agric. Food Chem.* **1988** *36*, 394-397
76. Tebbe, C. C.; Reber, H. H. *Appl. Microbiol. Biotechnol.* **1988** *29*, 103-105.
77. Smith, A. E. *J. Agric. Food Chem.* **1989** *37*, 267-271.
78. Duke, S. O. In *Herbicides: Chemistry, Degradation, and Mode of Action, Vol. 3,* Kearney, P. C.; Kaufmann, D. D., Eds.; Marcel Dekker, Inc., New York, 1988; pp. 1-70.
79. Pruess, D. L.; Scannell, J. P.; Blount, J. F.; Ax, H. A.; Kellett, M.; Williams, T. H.; Stempel, A. *J. Antibiot.* **1974** *27*, 754-759.
80. Ayer, S. W.; Isaac, B. G., Luchsinger, K.; Makkar, N.; Tran, M.; Stonard, R. J. *J. Antibiot.* **1991** *44*, 1460-1462.
81. Omura, S.; Murata, M.; Imamura, N.; Iwai, Y., Tanaka, H.; Furusaki, A.; Matasumuto, T. *J. Antibiot.* **1984** *37*, 1324-1332.
82. Babczinski, P.; Dorgerloh, M.; Löbberding, A.; Santel, H.-J.; Schmidt, R. R.; Schmitt, P.; Wünsche, C. *Pestic. Sci.* **1991** *33*, 439-446.
83. Fushimi, S.; Nishikawa, S.; Mito, N.; Ikemoto, M.; Sasaki, M.; Seto, H. *J. Antibiot.* **1989** *42*, 1370-1378.
84. Yamada, O.; Kaise, Y.; Futatsuya, F.; Ishida, S.; Ito, K.; Yamamoto, H.; Munakata, K. *Agric. Biol. Chem.* **1972** *36*, 2013-2015.
85. Omura, S.; Iwai, Y.; Takahashi, Y.; Sadakane, N.; Nakagawa, A.; Oiwa, H.; Hasegawa, Y. *J. Antibiot.* **1979** *32*, 255-261.
86. Arai, M; Haneishi, T.; Kitahara, N.; Enokita, R.; Kawakubo, K.; Kondo, Y.; *J. Antibiot.* **1976** *29*, 863-869.
87. Haneishi, T.; Terahara, A.; Kayamori, H.; Yabe, J.; Arai, M. *J. Antibiot.* **1976** *29*, 870-875.
88. Takiguchi, Y.; Yoshikawa, H.; Terahara, A.; Torikata, A.; Terao, M. *J. Antibiot.* **1979** *32*, 857-861.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

89. Nakajima, M.; Itoi, K.; Takamatsu, Y.; Kinoshita, T.; Okazaki, T.; Kawakubo, K.; Shindo, M.; Honma, T.; Tohjigamori, T. *J. Antibiot.* **1991** *44*, 293-300.
90. Heisey, R. M.; Putnam, A. R. *J. Nat. Prod.* **1986**, *49*, 859-865.
91. Heisey, R. M.; Putnam, A. R. *J. Plant Growth Regulat.* **1990**, *9*, 19-25.
92. Isaac, B. G.; Ayer, S. W.; Stonard, J. R. *J. Antibiot.* **1991** *44*, 795-796.
93. Nakajima, M.; Itoi, K.; Takamatsu, Y.; Sato, S.; Furukawa, Y.; Furuya, K.; Honma, T.; Kadotani, J.; Kozasa, M.; Haneishi, T. *J. Antibiot.* **1991** *44*, 1065-1072.
94. Strobel, G.; Stierle, A.; Park, S.H.; Cardellina, J. *Amer. Chem. Soc. Symp. Ser.* **1990** *439*, 53-52.
95. Mitchell, R. E.; Bieleski, R. L. *Plant Pathol.* **1977** *19*, 227-235.
96. Patil, S. S.; Walker, J. C. *Phytopathology* **1963** *53*, 522-528.
97. Rudolph, K.: Stahmann, M. A. *Phytopathology* **1966** *57*, 29-46
98. Mitchell, R. E. *Phytochemistry* **1976** *15*, 1941-1947.
99. Moore, R. E.; Niemczura, W. P. N.; Kwok, O. C. H.; Patil, S. S. *Tetrahedron Lett.* **1984** *25*, 3931-3934.
100. Ferguson, A. R.; Johnston, J. S. *Physiol. Plant Pathol.* **1980** *16*, 269-275.
101. Kwok, O. C. H.; Patil, S. S. *FEMS Microbiol. Lett.* **1982** *14*, 247-249.
102. Staskawicz, B. J.; Panopoulos, N. J. *J. Bacteriol.* **1982** *142*, 720-723.
103. Patil, S. S.; Tam, L. Q.; Kakai, W. S. *Plant Physiol.* **1972** *49*, 803-807.
104. Tam, L. Q.; Patil, S. S. *Plant Physiol.* **1972** *49*, 808-812.
105. Thomas, M. D.; Langston-Unkefer, P. L.; Uchytil, T. F.; Durbin, R. D. *Plant Physiol.* **1983** *71*, 912-915.
106. Langston-Unkefer, P. L.; Macy, P. A.; Durbin, R. D. *Plant Physiol.* **1984** *76*, 71-74.
107. Thomas, M. D.; Durbin, R. D. *J. Gen. Microbiol.* **1985** *131*, 1061-1067.
108. Uchytil, T. F.; Durbin, R. D. *Experientia* **1980** *36*, 301-302.
109. Durbin, R. D.; Uchytil, T. F. *Physiol. Plant Pathol.* **1984** *24*, 25-31.
110. Durbin, R. D.; Uchytil, T. F. *Experientia* **1985** *41*, 136-137.
111. Levi, C.; Durbin, R. D. *Physiol. Molec. Plant Pathol.* **1986** *28*, 345-352.
112. Lax, A. R.; Shepherd, H. S.; Edwards, J. V. *Weed Technol.* **1988** *2*, 540-549.
113. Vaughn, K. C.; Duke, S. O. *Physiol. Plant.* **1984** *60*, 257-261.
114. Steele, J. A.; Uchytil, T. F.; Durbin, R. D.; Bhatnagar, P.; Rich, D. H. *Proc. Nat. Acad. Sci. U. S. A.* **1976** *73*, 2245-2248.
115. Rich, D. H. In *Toxins in Plant Disease. Mechanism of Action;* Durbin, R. D., Ed.; Academic Press, New York, 1981. pp. 295-329.
116. Edwards, J. V.; Dailey, O. D.; Bland, J. M.; Cutler, H. G. *Amer. Chem. Soc. Symp. Ser.* **1988** *380*, 35-56.
117. Lax, A. R.; Shepherd, H. S. *Amer. Chem. Soc. Symp. Ser.* **1988** *380*, 24-34.
118. Duke, S. O.; Gohbara, M.; Paul, R. N.; Duke, M.V. *J. Phytopath.* **1992** *134*, 289-305.
119. Abbas, H. K.; Paul, R. N.; Boyette, C. D.; Duke, S. O.; Vesonder, R. F. *Can. J. Bot.* - In Press.
120. Gelderblom, W. C. A.; Jaskiewicz, K., Marasas, W. F. O.; Thiel, P. G.; Horak, R. M.; Vegglaar, R.; Kriek, N. P. J. *Appl. Environ. Microbiol.* **1988** *54*, 1806-1811.
121. Wang, E.; Norred, W. P.; Bacon, C. W.; Riley, R. T.; Merrill, A. H. *J. Biol. Chem.* **1991** *266*, 14486-14490.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

122. Gilchrist, D. G. In *Toxins and Plant Pathogenesis*, Daly, J. M.; Deverall, B. D., Eds., Academic Press, Sydney, 1983, pp. 81-136.
123. Fuson, G. B.; Pratt, D. *Phytopathol.* **1988** 78, 1641-1648.
124. Mirocha, C. J.; Gilchrist, D. G.; Shier, W. T.; Abbas, H. K.; Wen, Y.; Vesonder, R. F. *Mycopathologia* **1992** 117, 47-56.
125. Gilchrist, D. G.; Ward, B.; Moussato, V.; Mirocha, C. J. *Mycopathologia* **1992** *117*, 57-64.
126. Cutler, H. G. *CRC Crit. Rev. Plant Sci.* **1988** *6*, 323-343.
127. Nishimura, S.; Kohmoto, K. *Annu. Rev. Phytopathol.* **1983** *21*, 87-116.
128. Munakata, K.; Yamada, O.; Ishida, S.; Futatsuya, F.; Ito, I.; Yamamoto, H. *Proc. Asian-Pacific Weed Sci. Soc. Conf.* **1973** *4*, 1135-1136.
129. Murakami, T.; Anzai, H.; Imai, S.; Satoh, A.; Nagaoka, K.; Thompson, C. J. *Mol. Gen. Genet.* **1986** *205*, 42-50.
130. Edwards, J. V.; Lax, A. R.; Lillehoj, E. B.; Boudreaux, G. J. *J. Agric. Food Chem.* **1988** *35*, 451-456.
131. Miliszkiewicz, D.; Wieczorek, P.; Lejczak, B.; Kowalik, E.; Kafarski, P. *Pestic. Sci.* **1992** *34*, 349-354.
132. Wright, K.; Pillmoor, J. B.; Briggs, G. G. In *Topics in Photosynthesis-Vol. 10, Herbicides*; Baker, N. R.; Percival, M. P., Eds.; Elsevier Science Publishers, Amsterdam, 1991; pp. 337-368.
133. Lydon, J.; Duke, S. O. *Pestic. Sci.* **1989** *25*, 361-373.

RECEIVED July 22, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch009

Chapter 10

Insecticide Binding Sites on γ-Aminobutyric Acid Receptors of Insects and Mammals

Jon E. Hawkinson and John E. Casida

Pesticide Chemistry and Toxicology Laboratory, Department of Entomological Sciences, University of California, Berkeley, CA 94720

The γ-aminobutyric acid (GABA) receptor of insects and mammals has two binding sites or domains that are sensitive to nanomolar levels of selected highly effective insecticides. One of these is the noncompetitive blocker (NCB) site where the polychlorocycloalkanes (PCCAs), picrotoxinin (PTX), 2,6,7-trioxabicyclo[2.2.2]octanes (TBOs), and dithianes act to block the integral chloride channel of the GABA receptor complex. The other site interacts with insecticidal GABA-gated chloride channel activators exemplified by avermectin B_{1a} (AVM). Binding studies with [^{3}H]TBOs, [^{3}H]AVM, and radioligands specific for other sites on the receptor complex as well as chloride flux measurements serve to define the properties of the binding sites, the coupling between the NCB, AVM, and other sites, the nature of target site alterations in resistant strains, and the contribution of species-dependent receptor diversity to selective toxicity.

The three primary targets for insecticide action are the GABA-gated chloride channel, the voltage-dependent sodium channel, and the cholinergic system. This review considers the first of these targets with particular attention to binding sites for several classes of potent insecticides and comments on applying this knowledge to safer insecticides for the future.

The GABAergic System

GABA is the principal inhibitory neurotransmitter of the central nervous system (CNS) of mammals and insects and the neuromuscular junction of insects. Two types of receptors mediate the inhibitory responses of GABA in mammals. Baclofen-sensitive $GABA_B$ receptors are coupled to calcium and potassium channels via G-proteins (*1*), whereas $GABA_A$ receptors are members of the super-family of ligand-gated ion channels containing an integral chloride channel (*2*). A nerve

0097–6156/93/0524–0126$06.00/0

impulse reaching an inhibitory synapse releases GABA presynaptically which diffuses across the synapse and binds to the postsynaptic $GABA_A$ receptor, resulting in chloride entry, hyperpolarization, and inhibition of the postsynaptic neuron. The $GABA_A$ receptor of mammalian brain consists of four or five 50 to 60 kD glycoprotein subunits (Figure 1) for which five classes and several sub-types have been cloned from rat, bovine, human, and/or chicken brain (or retina), including 6α, 4β, 3γ, 1δ, and 1ρ (*3*). There is a high degree of amino acid sequence identity (60-80%) between subtypes within the same class, and a lower degree of sequence identity (20-40%) between different subunit classes (*4*). A cyclodiene resistance-conferring gene from *Drosophila* (*5*) and a functional mollusc GABA receptor subunit (*6*) have been cloned and show homology with the mammalian brain β subunit.

Knowledge of the GABA-modulated chloride ionophore has developed rapidly in the past decade, spurred by pharmaceutical and pesticide research. Compounds that facilitate GABA-mediated inhibition have sedative/anxiolytic/anticonvulsant/muscle relaxant properties [benzodiazepines (BZDs) and barbiturates] whereas those that antagonize GABA (bicuculline) or are chloride channel blockers (PTX, PCCAs, TBOs) are convulsants. Electrophysiological systems useful in studying GABAergic neurotransmission include GABA-induced chloride currents in insect, nematode, and crustacean muscle, vertebrate spinal cord and hippocampal neurons, cultured insect and mammalian neurons, and *Xenopus* oocytes injected with RNA or expressing cloned receptors. Insect neuronal GABA receptors show similarities to the mammalian $GABA_A$ receptor in that they are GABA-activated, baclofen-insensitive chloride channels, potentiated by BZDs and barbiturates and blocked by PTX (*7, 8*). However, larger anions can permeate the insect channel (*9*) and the potency order for agonists differs between insect and mammalian receptors. Morever, potent competitive antagonists in the mammal such as bicuculline are inactive in the insect. Cockroach muscle GABA-gated chloride channels display similar properties to neuronal receptors in that they are activated by GABA or muscimol, and are noncompetitively blocked by PTX. However, the efficacy of GABA agonists in insect muscle is different than for nerve receptors and the action of bicuculline is conflicting (*10, 11*).

Two *in vitro* physiological systems allow the study of the relationship between GABA, chloride ion, modulators and inhibitors. The first measures GABA-induced ^{36}chloride flux in invertebrate muscle fibers or insect and mammalian brain microvesicles. Chloride flux in mammalian preparations is stimulated by GABA and avermectins (AVMs) (*12*). This GABA-stimulated chloride flux is enhanced by barbiturates (*13*) and BZDs (*14*) and inhibited by bicuculline and channel blockers such as PTX, PCCAs and TBOs (*15*). Similar chloride flux is observed in invertebrates except bicuculline is weakly antagonistic (*16*) or inactive (*17*). The second *in vitro* system measures ivermectin-(22,23-dihydro-AVM or IVM)-stimulated neurotransmitter release from cockroach CNS synaptosomes following preloading with [^{3}H]choline; the released material is presumed to be [^{3}H]acetylcholine. This release is inhibited by PTX and other chloride channel blockers (*18, 19*). An alternative approach for characterizing the GABAergic system involves examination of the binding sites with selected radioligands.

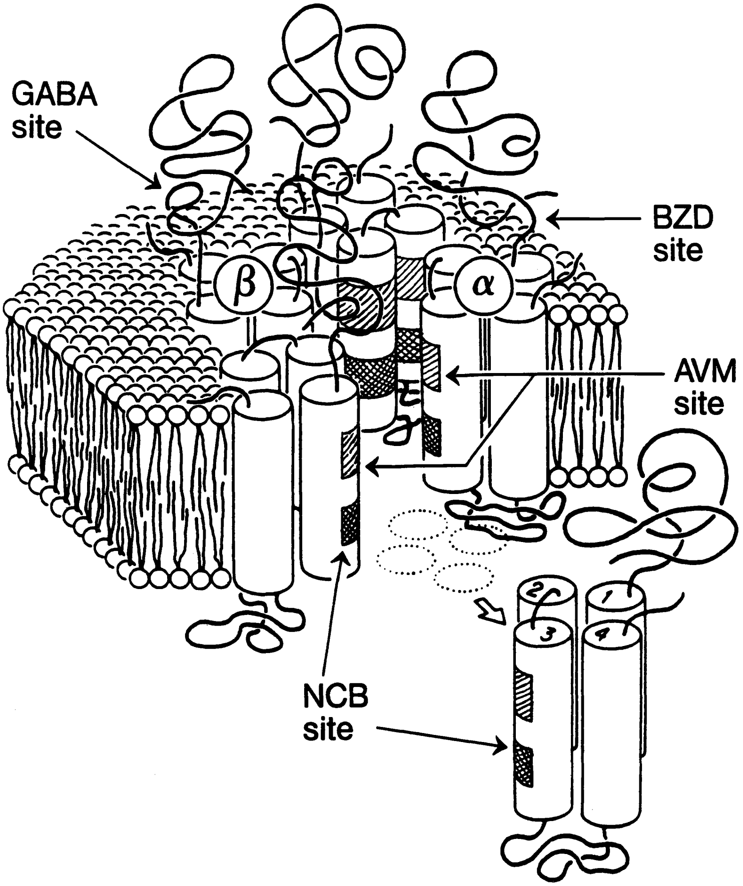


Figure 1. Suggested Structure of $GABA_A$ Receptor with Possible Locations of GABA, Benzodiazepine, Noncompetitive Blocker, and Avermectin Binding Sites. The localizations of the GABA and BZD sites are consistent with current findings whereas those of the NCB and AVM sites are speculative. Adapted from ref. 4.

Binding Sites for GABA, Benzodiazepines, and Barbiturates (Figure 1)

The GABA binding site is most important in channel gating. The mammalian site can be assayed using the agonists [^{3}H]GABA and the more potent [^{3}H]muscimol or the competitive antagonists [^{3}H]bicuculline methiodide and the more potent [^{3}H]SR 95531. [^{3}H]Agonists are preferentially displaced by agonists and [^{3}H]antagonists are preferentially displaced by antagonists. The corresponding insect site has a different structure-activity relationship (SAR) for agonists whereas bicuculline is inactive (*20*).

The central BZD site exhibits a rich pharmacology in as much as agonists (depressants), inverse agonists (convulsants), and antagonists (no intrinsic action but block the action of agonists and inverse agonists) modulate GABAergic transmission in the mammalian CNS. Agonist, antagonist, and inverse agonist radioligands include the benzodiazepine [^{3}H]flunitrazepam, the imidazodiazepine [^{3}H]Ro 15-1788, and the β-carboline [^{3}H]β-CCE, respectively. A similar site apparently exists on insect GABA receptors, although it has a pharmacological profile similar to the peripheral BZD site which is not associated with GABA receptors in mammals (*20, 21*). Additional sites exist on the receptor complex for which there are no suitable radioligands. In particular, no barbiturate binding site has been detected directly. The facilitory actions of barbiturate agonists are evaluated by their effects on the binding of ligands to other sites on the receptor complex.

Noncompetitive Blockers (Figures 2 and 3)

The epoxylactone PTX from the fishberry plant (*Anamirta cocculus*) is toxic due to noncompetitive block of GABA-induced chloride ion conductance (*22*). Related botanicals may act in the same manner, e.g. picrotin and anisatin (from *Illicium anisatum*). Other proposed noncompetitive GABA antagonists are argophyllin A from sunflower (*Helianthus annuus*) (*23*) and aplysiaterpenoid A from a marine alga (*Plocamium telfairiae*) (*24*). Attempts to improve on the potency of PTX by structural variations have generally proven unsuccessful. Some synthetic lactones also modulate GABA receptor function. The change from a β- to an α-alkyl-substituted γ-butyrolactone or more potent γ-butyrothiolactone can change a convulsant to a depressant, both apparently acting at the NCB site but in opposite manners (*25*). The corresponding *bis*-thiololactone is a convulsant with moderate activity in the mammalian receptor preparation (*26*).

The PCCAs are the most important chloride channel blockers from the standpoint of pest control and environmental impact. About 1 billion (1 x 10^9) pounds of lindane and its isomers were used as insecticides and another billion of the polychlorobornane insecticide toxaphene, and yet a third billion of the cyclodienes such as dieldrin and endosulfan. These compounds are now heavily restricted although many uses continue for lindane and endosulfan, which have more favorable toxicological profiles than most of the compounds dropped from use. In rats, PCCAs block GABA-mediated inhibition in brain (*27*), GABA-

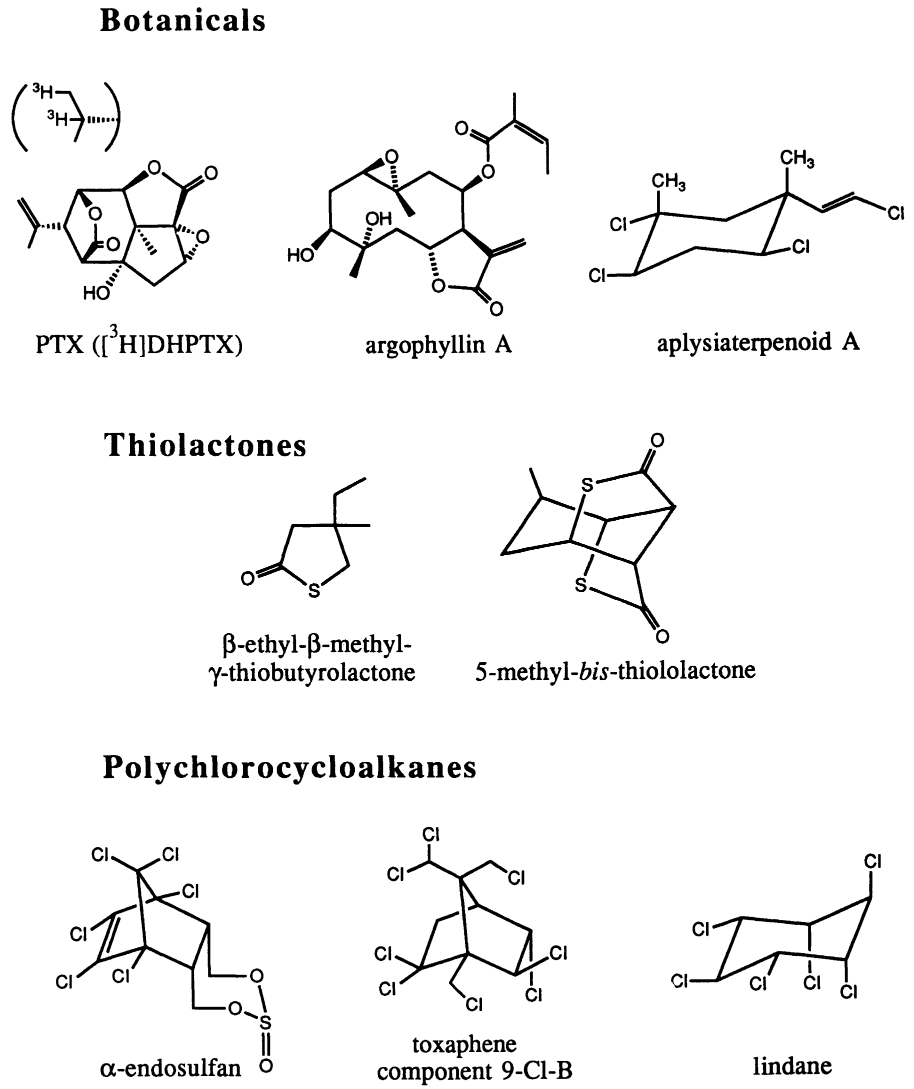
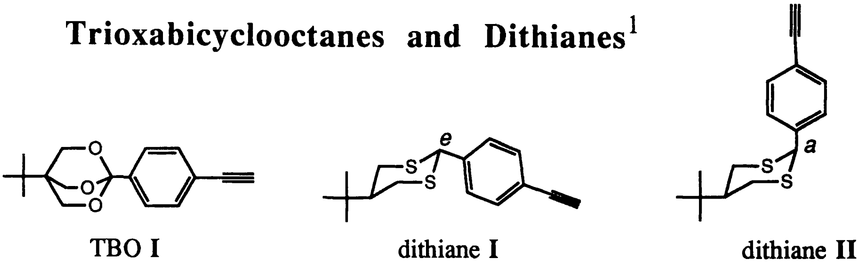
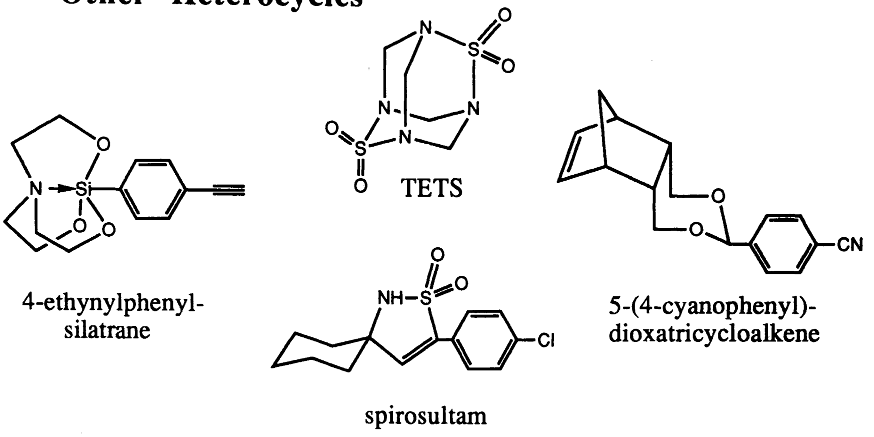

Botanicals
PTX ([^{3}H]DHPTX)
argophyllin A
aplysiaterpenoid A
Thiolactones
β-ethyl-β-methyl-γ-thiobutyrolactone
5-methyl-*bis*-thiololactone
Polychlorocycloalkanes
α-endosulfan
toxaphene component 9-Cl-B
lindane

Figure 2. Structures of Noncompetitive Chloride Channel Blockers: Botanicals, Thiolactones, and Polychlorocycloalkanes.

Trioxabicyclooctanes and Dithianes[1]


O
O
O
S
S
e
S
S
a
TBO I
dithiane I
dithiane II

Other Heterocycles


O
N
Si
O
O
4-ethynylphenyl-
silatrane
N
S
O
O
N
N
N
O
S
O
TETS
O
O
NH-S
Cl
spirosultam
O
O
CN
5-(4-cyanophenyl)-
dioxatricycloalkene

---

[1] *e* and *a* are equatorial and axial, resp.

Figure 3. Structures of Noncompetitive Chloride Channel Blockers: Trioxabicyclooctanes, Dithianes, and other Heterocycles.

activated chloride currents in cultured neurons (*28*), and GABA-stimulated chloride flux in brain microvesicles (*15*). Similarly in cockroaches, lindane and the cyclodienes are effective non-competitive blockers of GABA-activated currents and GABA-stimulated chloride flux in CNS and/or muscle (*29-31*).

The TBOs (*32, 33*) and dithianes (*34-36*) are highly potent insecticides for house flies and some other species and have similar structure-activity relationships as to the optimal substituents, e.g. *t*-butyl and ethynylphenyl moieties (Table I). Structural optimization of the TBOs and dithianes is at the point where the insecticidal potency of NCB site blockers rivals those of the most effective compounds acting at the cholinergic system and sodium channel. Compounds in these groups are by far the most potent NCB site convulsants in mammals and toxicants in insects. Optimal TBOs inhibit binding of NCB site radioligands (*32, 33*) and block GABA-stimulated chloride flux (*15*) and GABA-activated chloride currents (*37*) at nanomolar levels in vertebrates. In insects, TBOs block ivermectin-stimulated [^{3}H]acetylcholine release (*19*) and GABA-mediated muscle relaxation (*32*). These compounds provided the basis for development of several very effective radioligands discussed later. Less active blockers or insecticides are the silatranes (*38*), sulfamides such as tetramethylenedisulfotetramine (TETS) (*39*), dioxatricycloalkenes (*40*), and a spirosultam (*41*).

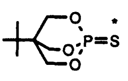
Table I. Insecticidal Activity and Insect and Mammalian Receptor Potency of TBOs and Dithianes with Appropriately Positioned *t*-Butyl and *p*-Ethynylphenyl Substituents and Comparison with Other Insecticides[a]

Insecticide	*House Fly LD$_{50}$ (µg/g)*		*Receptor IC$_{50}$ (nM)*	
			[^{3}H]EBOB	*[^{35}S]TBPS*
	Alone	*+PB*	*House Fly*	*Mouse*
TBO I[b]	0.090	0.011	3.9	1
dithiane I[c]	1.4	0.1	8.1	4
dithiane I sulfone	0.6	0.01	5.4	1
dithiane II	2.5	0.6	14	8
dithiane II sulfone	0.6	0.02	N.D.	1
parathion	0.21	0.012	N.D.	N.D.
[1R,cis]permethrin	1.3	0.43	N.D.	N.D.

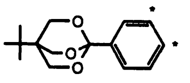
[a] See Figure 3 for stuctures of TBO and dithianes. Sources: Adapted from refs. 33, 36, 42. See also ref. 35. N.D. = not determined
[b] For related compounds see ref. 33.
[c] For related compounds see ref. 34.

Noncompetitive Blocker Site in Mammals

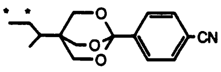
[^{3}H]Dihydropicrotoxinin ([^{3}H]DHPTX) (Figure 2), the first radioligand for the NCB site (*43*), had low specific activity, affinity, and specific binding. No [^{3}H]PCCA has been successfully used to date to characterize its binding site. The mammalian NCB site is readily assayed by binding of radiolabeled TBOs (shown below), including [^{35}S]TBPS (K_d ~20 nM) (*44, 45*), [^{3}H]TBOB (K_d ~60 nM) (*46*) and the high affinity (K_d 1-4 nM) [^{3}H]EBOB (Cole, L.M.; Casida, J.E. *Pestic Biochem. Physiol.*, in press). Chloride and other chloride-channel permeant anions stimulate binding. GABA and GABA mimetics inhibit binding of NCB site radioligands, an effect reversed by the potent competitive GABA antagonist R5135. GABA facilitators, such as depressant barbiturates and BZDs, also inhibit binding in the presence of GABA (*47*). The pharmacological profile for [^{35}S]TBPS binding established its association with the GABA$_A$ receptor (*44*), which has been confirmed in purified (*48*) and immunoprecipitated (*49*) receptor complexes. There are good correlations for compounds as inhibitors of [^{35}S]TBPS binding and GABA-induced chloride flux (*15*) establishing that affinity for the NCB site is a good indicator of chloride channel block. The NCB site is strongly conserved through the vertebrates in its binding parameters and pharmacological profiles examined with [^{35}S]TBPS for human, bovine, rat, chicken, and fish brain (*50*) and with [^{3}H]EBOB for human, dog, mouse, chicken, and fish brain (Cole, L.M.; Casida, J.E. *Pestic Biochem. Physiol.*, in press).



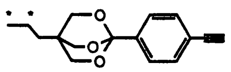
[^{35}S]TBPS



[^{3}H]TBOB



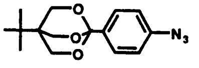
[^{3}H]CN*s*BOB



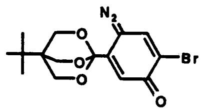
[^{3}H]EBOB

The binding kinetics of NCB site ligands at the bovine brain GABA$_A$ receptor provide new insight as to how they interact with the receptor complex (Hawkinson, J.E.; Casida, J.E., submitted). The binding kinetics were determined for unlabeled ligands by their effect on the [^{35}S]TBPS association curve in co-incubation experiments validated by comparisons of labeled and unlabeled TBOB, CNsBOB, and EBOB. The association rate governs the affinity of the TBOs (Figure 4) and dithianes, whereas the dissociation rate appears to be more important for the PCCAs. The low overall association rate suggests that NCB site ligands bind to a slowly-forming blocked receptor conformation.

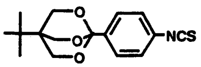
Localization of the NCB site as to receptor subunit and amino acid sequence remains unknown for lack of a suitable irreversible probe. Photoaffinity and chemical affinity probes which have been shown to irreversibly inhibit [^{3}H]TBOB or [^{35}S]TBPS binding are shown below



arylazido



diazocyclohexadienone



isothiocyanate

The efficacy of the photoaffinity probes is limited by low photoreactivity and/or affinity (*51*), whereas selective covalent modification of the receptor has not yet been demonstrated for the chemical affinity probe (*52*). Moreover, no radiolabeled affinity probe has yet been shown to covalently modify the binding site.

Noncompetitive Blocker Site in Insects

The insect NCB site was examined with each of the radioligands mentioned above. [^{3}H]DHPTX provided useful information (*53*) although it was far from an ideal probe. The pharmacological properties of [^{35}S]TBPS binding in house fly heads (*54*), thoraces plus abdomens (*55*), and locust ganglia (*56*) vary widely, particularly with respect to modulation by GABA, and the toxicological relevance of binding for this radioligand and the related [^{3}H]propyl bicyclophosphate (*57*) has not been demonstrated. No specific binding was observed for [^{3}H]TBOB in insects. [^{3}H]CNsBOB was an improved radioligand with cockroach CNS membranes but only limited data are available (*58*). [^{3}H]EBOB is the preferred radioligand for the NCB site of house fly and fruitfly heads (K_d 1-2 nM) since there is a good correlation in house flies between binding inhibition and toxicity for seven classes of NCB site blockers establishing the toxicological relevance of this binding assay (*42*) (Figure 5). Compared to mammals, the insect (house fly) NCB site is less sensitive to GABA agonists and chloride ion, is more sensitive to AVMs, and is insensitive to GABA facilitators and bicuculline (Table II).

Table II. Comparison of the NCB Sites of Mammals and Insects

	sensitivity of NCB site[a]	
modulator	*mammals*	*insects*
GABA	++	+
chloride	++	+
bicuculline	++	-
AVM	+	++
BZD	+	-
barbiturate	+	-
NCBs	++	++

[a] Measured with [^{35}S]TBPS, [^{3}H]TBOB or [^{3}H]EBOB in mammals and [^{3}H]EBOB in insects.

Insecticide affinity for the [^{3}H]EBOB binding site varies considerably between vertebrate and insect species. The insect site is more sensitive to a variety of channel blockers (cyclodienes, toxaphene, lindane, PTX, a dithiane) and especially to the AVMs (Table III). In addition to other possible mechanisms, these data suggest that receptor site specificity contributes to species selectivity. Despite these quantitative differences, the insect and mammalian binding sites must share a similar topography since, for a limited series of structurally-related compounds, there are good correlations between mouse brain receptor potency and housefly toxicity, shown for a subset of dithianes in Figure 6.

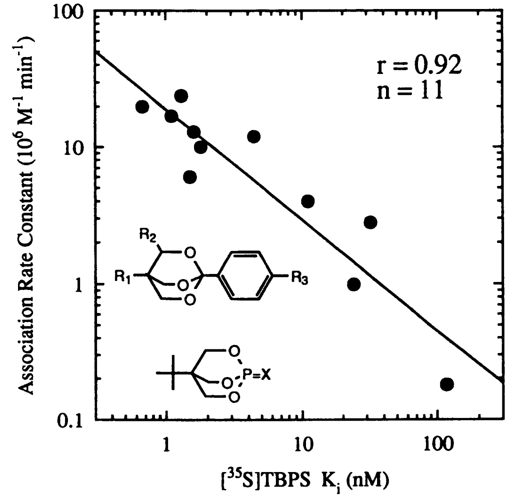


Figure 4. Correlation of Association Rate and Inhibition Constants for TBOs. Association rate constants for unlabeled TBOs estimated from their effect on the control [^{35}S]TBPS association time course in co-incubation experiments using bovine brain membranes. Inhibition constant (K_i) calculated from standard [^{35}S]TBPS competition experiments. Adapted from Hawkinson, J.E.; Casida, J.E., submitted.

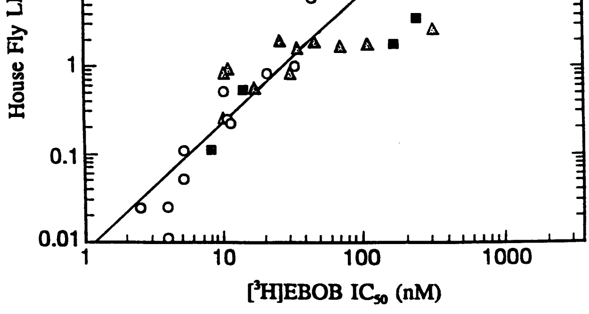


Figure 5. Correlation for Seven Classes of Chloride Channel Blockers as Inhibitors of [^{3}H]EBOB Binding and Toxicity in House Flies. [^{3}H]EBOB binding to house fly head membranes from sensitive strain. House flies pretreated with piperonyl butoxide for toxicity determinations. Inactive compounds (IC_{50} > 2000 nM, LD_{50} > 500 µg/g) are shown in the upper right box. Adapted from ref. 42.

Table III. [^{3}H]EBOB Binding Site Selectivity Ratios

≤0.2	TBPS, 4-*t*-butyl-1-ethynyl-TBO
2-8	cyclodienes, toxaphene, PTX, 5-*t*-butyl-2*e*-(4-bromophenyl)-dithiane
99	lindane
>600	AVM, moxidectin

Average IC_{50} for receptors from hen, dog, mouse, and chicken brains divided by IC_{50} for house fly head membranes (and in some cases fruitfly head membranes). Source: Adapted from Cole, L.M.; Casida, J.E. *Pestic. Biochem. Physiol.*, in press.

PCCA (dieldrin)-resistant insect strains exhibit reduced binding of NCB site ligands. Large decreases in both the affinity and binding capacity of [^{3}H]DHPTX to resistant cockroach brain membranes indicate that target site insensitivity is the main mechanism of cyclodiene resistance in insects (*53*) which has also been shown in electrophysiological experiments (*59, 60*). In a resistant strain of house flies, reduced [^{3}H]EBOB binding is due solely to reduced affinity for the binding site (*42*) (Figure 7). In contrast, no difference in [^{35}S]TBPS binding was observed between cyclodiene-resistant and -susceptible house fly strains (*61*) further indicating that this ligand is not appropriate for the insect NCB site. Cross-resistance is evident in house flies for each of the seven insecticide classes indicated above suggesting that the structural modifications in the receptor underlying the reduced [^{3}H]EBOB affinity are the same for all of these insecticides. A comparison of the receptor cloned from susceptible and resistant *Drosophila* strains may identify the sequence modification responsible for resistance. In resistant mosquitofish brain membranes, the reduction in [^{35}S]TBPS binding was also largely due to reduced affinity for the site (*62*).

Avermectin Binding Site in Mammals, Insects, and Nematodes

The remarkable potency of the AVMs led to their very effective and extensive use as insecticides, miticides, and anthelmintics (e.g. for treatment of onchocerciasis or African river blindness in man) (*63*). The 4″-deoxy-4″-*epi*(methylamino) analog of AVM has greatly increased lepidopterous larvicidal activity. The dioleandrosyl substituent of AVM is not present in closely-related macrocyclic lactones such as the highly miticidal moxidectin. The presence or absence of the sugar moiety greatly influences species specificity.

Three major types of action have been described for the AVMs. First, they open neurotransmitter-gated chloride channels associated with GABA receptors in vertebrates (*12, 64-66*), GABA and/or glutamate receptors in insect muscle (*67, 68*), and the multitransmitter-gated channel in crustacea (*69*). Secondly, AVMs block GABA-stimulated chloride channels in various preparations from mammals (*12, 64, 70*), and in locust (*67*) and nematode (*71, 72*) muscle. Thirdly, they open

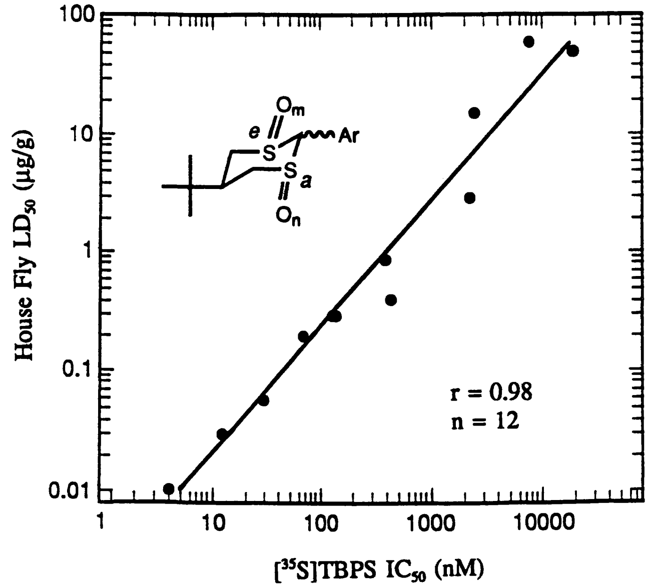


Figure 6. Correlation for Dithianes as Inhibitors of Mouse Brain [^{35}S]TBPS Binding and Toxicity to House Flies. Linear regression for dithiane S-oxides containing at least one oxygen atom *cis* to the aryl group (Ar), where Ar is 4-bromophenyl, 4-ethynylphenyl, or phenyl. For equatorial Ar, m=0 n=1 or m=n=1 and for axial Ar, m=1 n=0 or m=n=1. Adapted from ref. 36.

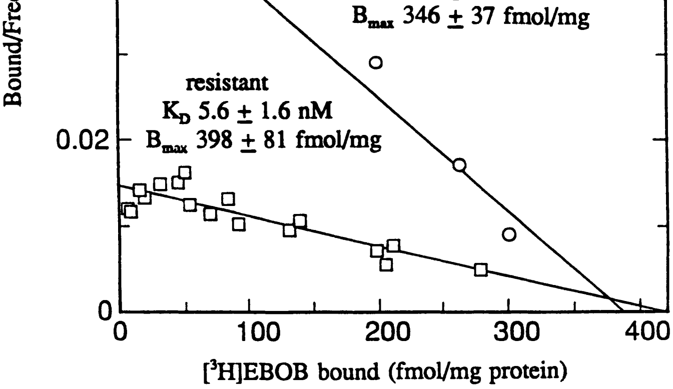
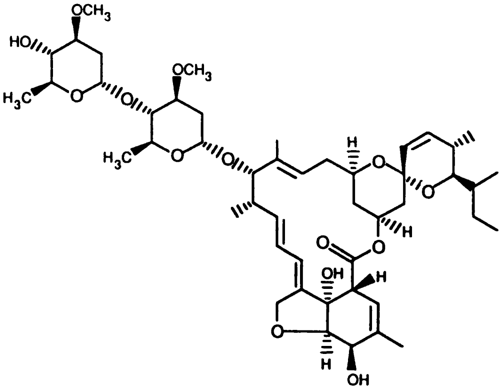
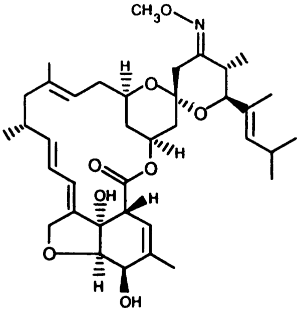


Figure 7. Scatchard Plot of [^{3}H]EBOB Binding to House Fly Head Membranes from Sensitive and Dieldrin-Resistant Strains. Adapted from ref. 42.

apparently non-neurotransmitter-gated chloride channels in mammals (*12, 73*), insects (*67, 74, 75*), and nematodes (*72, 76*).



avermectin B_{1a}



moxidectin

The effects of AVMs on GABA receptor ligands suggest an action at the GABA-gated chloride channel. AVM modulates the binding of GABA site ligands to insect CNS membranes (*77, 78*) and also GABA and benzodiazepine site ligands to rat brain membranes (*12, 79-84*). Although the AVMs may have multiple sites of action, they probably act primarily (i.e. at lower concentrations) as GABA-gated chloride channel activators in mammals; their apparent inhibitory action may be due to a lower efficacy than GABA as channel openers. In nematodes, AVMs act primarily as activators of chloride channels which may not be gated by neurotransmitters.

[^{3}H]AVM and [^{3}H]IVM exhibit specific, high affinity and saturable binding in membrane preparations from mammalian brain (*85, 86*), house fly heads (Deng, Y.L.; Casida, J.E. ***Pestic. Biochem. Physiol.***, in press) and crude (*87*) as well as solublilized (*88*) nematode membranes. The binding affinity is about 10-fold higher in nematodes (K_d 0.1-0.2 nM) than in mammals or insects (K_d 1-2 nM). [^{3}H]AVM binding is toxicologically relevant for the AVM analogs based on their potency as displacers of [^{3}H]AVM binding compared with inhibition of motility in nematodes (*87*) or toxicity to house flies (Deng, Y.L.; Casida, J.E. ***Pestic. Biochem. Physiol.***, in press) (Figure 8).

The AVM binding protein has been difficult to identify since GABA (or other putative neurotransmitters) and a variety of NCB site blockers generally have no effect on [^{3}H]AVM binding. However, GABA agonists inhibit and NCB site ligands stimulate binding of [^{3}H]AVM to rat brain membranes in the absence of chloride ion (*86*). The nature of the interaction of the AVM and NCB sites on GABA receptors in mammalian brain is unclear since the effect of AVM on [^{35}S]TBPS binding ranges from stimulation (*89*) to biphasic (*80*) to inhibition only (*12*). In contrast, the AVM site is closely coupled to the GABA receptor NCB site in house fly (Deng, Y.L.; Casida, J.E. ***Pestic. Biochem. Physiol.***, in press) and fruitfly (Cole, L.M.; Casida, J.E. ***Pestic. Biochem. Physiol.***, in press) preparations. AVM analogs have the same SAR for inhibition of [^{3}H]AVM and [^{3}H]EBOB

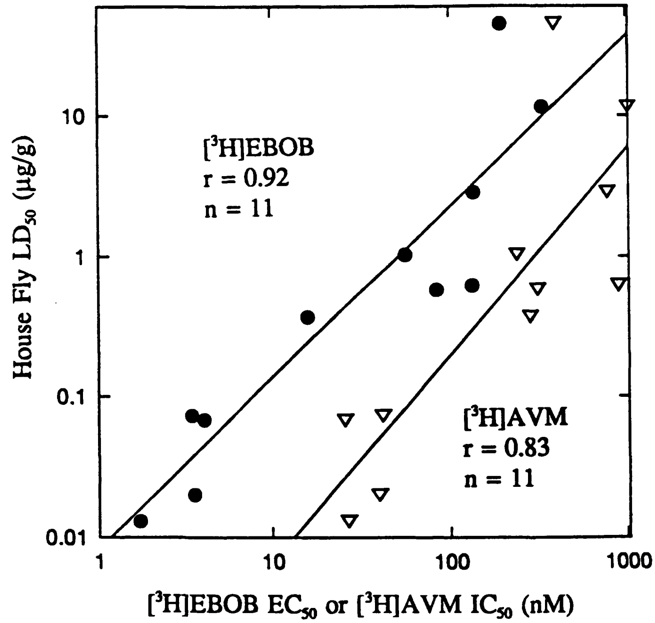


Figure 8. Correlation for AVMs as Inhibitors of [^{3}H]AVM and [^{3}H]EBOB Binding and Toxicity in House Flies. [^{3}H]EBOB and [^{3}H]AVM binding to house fly head membranes from sensitive strain. House flies pretreated with piperonyl butoxide for toxicity determinations. Adapted from Deng, Y.L.; Casida, J.E.; *Pestic. Biochem. Physiol.*, in press.

binding in house fly head membranes (Figure 8) verifying the close coupling between their binding sites and providing strong evidence for the action of AVM analogs at the GABA-gated chloride channel in insects. However, the AVM and NCB sites are sufficiently distinct in dieldrin-resistant house flies that there is no cross resistance (*42*). Thorax membranes from AVM-resistant flies have a decreased number of binding sites rather than an altered receptor affinity (*90*).

Future of GABA Receptors as Insecticide Targets

GABA receptors were recognized only a decade ago as sensitive targets for insecticide action. PTX had already been known for nearly a century as a minor use insecticide before its action at the GABA receptor was discovered. The PCCAs were well established (3 billion pounds used) for a half century before they were shown to also act as blockers of the GABA-gated chloride channel. The AVMs span this last decade with rapid advances in their development and use as chloride channel activators.

Future goals for insecticides acting at the GABA receptor include improved agents and knowledge of their molecular toxicology. The channel blockers are increasingly noted for structural diversity including a wide range of cycloalkyl and heterocyclic compounds. Improvements are needed in the spectrum of insecticidal activity and in reduced toxicity to mammals and fish. Resistance due to a modified NCB site is a potential problem now or in the future and care is required to minimize this possibility with reinstatement of selection pressure. The AVMs are undergoing progressive optimization for structural simplification, potency and selectivity.

The GABAergic system is one of the most sensitive targets for insecticide action, allowing very potent insecticides often with suitable selective properties. It is for now an underutilized target and therefore a challenge for future developments.

Acknowledgments

We thank our laboratory colleagues Loretta Cole and Yanli Deng for helpful discussions. Supported in part by National Institutes of Health Grant PO1 ES0009.

Literature Cited

1. Bowery, N. *Trends Pharmacol. Sci.* **1989**, *10*, 401-407.
2. Schofield, P.R.; Darlison, M.G.; Fujita, N.; Burt, D.R.; Stephenson, F.A.; Rodriguez, H.; Rhee, L.M.; Ramachandran, J.; Reale, V.; Glencorse, T.A.; Seeburg, P.H.; Barnard, E.A. *Nature* **1987**, *328*, 221-227.
3. Burt, D.R.; Kamatchi, G.L. *FASEB J.* **1991**, *5*, 2916-2923.
4. Olsen, R.W.; Tobin, A.J. *FASEB J.* **1990**, *4*, 1469-1480.
5. ffrench-Constant, R.H.; Mortlock, D.P.; Shaffer, C.D.; MacIntyre, R.J.; Roush, R.T. *Proc. Natl. Acad. Sci. (USA)* **1991**, *88*, 7209-7213.
6. Harvey, R.J.; Vreugdenhil, E.; Zaman, S.H.; Bhandal, N.S.; Usherwood, P.N.R.; Barnard, E.A.; Darlison, M.G. *EMBO J.* **1991**, *10*, 3239-3245.

7. Lees, G.; Beadle, D.J.; Neumann, R.; Benson, J.A. *Brain Res.* **1987**, *401*, 267-278.
8. Sattelle, D.B.; Pinnock, R.D.; Wafford, K.A.; David, J.A. *Proc. R. Soc. Lond.* **1988**, *B232*, 443-456.
9. Pinnock, R.D.; David, J.A.; Sattelle, D.B. *Proc. R. Soc. Lond.* **1988**, *B232*, 457-470.
10. Scott, R.H.; Duce, I.R. *Comp. Biochem. Physiol.* **1987**, *86C*, 305-311.
11. Murphy, V.F.; Wann, K.T. *Br. J. Pharmacol.* **1988**, *95*, 713-722.
12. Abalis, I.M.; Eldefrawi, A.T.; Eldefrawi, M.E. *J. Biochem. Toxicol.* **1986**, *1*, 69-82.
13. Harris, R.A.; Allan, A.M. *Science* **1985**, *228*, 1108-1110.
14. Morrow, A.L.; Paul, S.M. *J. Neurochem.* **1988**, *50*, 302-306.
15. Obata, T.; Yamamura, H.I.; Malatynska, E.; Ikeda, M.; Laird, H.; Palmer, C.J.; Casida, J.E. *J. Pharmacol. Exp. Ther.* **1988**, *244*, 802-806.
16. Ticku, M.K.; Olsen, R.W. *Biochim. Biophys. Acta.* **1977**, *464*, 519-529.
17. Wafford, K.A.; Sattelle, D.B.; Abalis, I.; Eldefrawi, A.T.; Eldefrawi, M.E. *J. Neurochem.* **1987**, *48*, 177-180.
18. Nicholson, R.A.; Baines, P.; Robinson, P.S. *Am. Chem. Soc. Symp. Ser.* **1987**, *356*, 262-272.
19. Nicholson, R.A.; Robinson, P.S.; Palmer, C.J.; Casida, J.E. *Pestic. Sci.* **1988**, *24*, 185-187.
20. Lummis, S.C.R.; Sattelle, D.B. *Neurochem. Int.* **1986**, *9*, 287-293.
21. Robinson, T.; MacAllan, D.; Lunt, G.; Battersby, M. *J. Neurochem.* **1986**, *47*, 1955-1962.
22. Takeuchi, A.; Takeuchi, N. *J. Physiol. (Lond.)* **1969**, *205*, 377-391.
23. Mullin, C.A.; Alfatafta, A.A.; Harman, J.L.; Everett, S.L.; Serino, A.A. *J. Agric. Food Chem.* **1991**, *39*, 2293-2299.
24. Watanabe, K.; Umeda, K.; Kurita, Y.; Takayama, C.; Miyakado, M. *Pestic. Biochem. Physiol.* **1990**, *37*, 275-286.
25. Holland, K.D.; McKeon, A.C.; Covey, D.F.; Ferrendelli, J.A. *J. Pharmacol. Exp. Ther.* **1990**, *254*, 578-583.
26. Kawamura, N.; Casida, J.E. *Chem. Res. Toxicol.* **1990**, *3*, 453-457.
27. Joy, R.M.; Albertson, T.E. *Neurotoxicol.* **1987**, *8*, 529-542.
28. Ogata, N.; Vogel, S.M.; Narahashi, T. *FASEB J.* **1988**, *2*, 2895-2900.
29. Ghiasuddin, S.M.; Matsumura, F. *Comp. Biochem. Physiol.* **1982**, *73C*, 141-144.
30. Lummis, S.C.R.; Buckingham, S.D.; Rauh, J.J.; Sattelle, D.B. *Proc. R. Soc. Lond.* **1990**, *B240*, 97-106.
31. Wafford, K.A.; Lummis, S.C.R.; Sattelle, D.B. *Proc. R. Soc. Lond.* **1989**, *B237*, 53-61.
32. Casida, J.E.; Palmer, C.J.; Cole, L.M. *Mol. Pharmacol.* **1985**, *28*, 246-253.
33. Palmer, C.J.; Cole, L.M.; Larkin, J.P.; Smith, I.H.; Casida, J.E. *J. Agric. Food Chem.* **1991**, *39*, 1329-1334.
34. Elliott, M.; Pulman, D.A.; Larkin, J.P.; Casida, J.E. *J. Agric. Food Chem.* **1992**, *40*, 147-151.
35. Palmer, C.J.; Casida, J.E. *J. Agric. Food Chem.* **1992**, *40*, 492-496.
36. Wacher, V.J.; Toia, R.F.; Casida, J.E. *J. Agric. Food Chem.* **1992**, *40*, 497-505.

37. Van Renterghem, C.; Bilbe, G.; Moss, S.; Smart, T.G.; Constanti, A.; Brown, D.A.; Barnard, E.A. *Mol. Brain Res.* **1987**, *2*, 21-31.
38. Horsham, M.A.; Palmer, C.J.; Cole, L.M.; Casida, J.E. *J. Agric. Food Chem.* **1990**, *38*, 1734-1738.
39. Esser, T.; Karu, A.E.; Toia, R.F.; Casida, J.E. *Chem. Res. Toxicol.* **1991**, *4*, 162-167.
40. Ozoe, Y.; Sawada, Y.; Mochida, K.; Nakamura, T.; Matsumura, F. *J. Agric. Food Chem.* **1990**, *38*, 1264-1268.
41. Gajewski, R.P.; Jackson, J.L.; Karr, L.L.; Bloomquist, J.R. *Abstracts Neurotox. '91: An International Symposium on the Molecular Basis of Drug and Pesticide Action.* Univ. Southampton, U.K., **1991**; 61-62.
42. Deng, Y.; Palmer, C.J.; Casida, J.E. *Pestic. Biochem. Physiol.* **1991**, *41*, 60-65.
43. Ticku, M.K.; Ban, M.; Olsen, R.W. *Mol. Pharmacol.* **1978**, *14*, 391-402.
44. Squires, R.F.; Casida, J.E.; Richardson, M.; Saederup, E. *Mol. Pharmacol.* **1983**, *23*, 326-336.
45. Squires, R.F. Ed., *GABA and Benzodiazepine Receptors* Volumes I and II; CRC Press: Boca Raton, Florida, **1988**.
46. Lawrence, L.J.; Palmer, C.J.; Gee, K.W.; Wang, X.; Yamamura, H.I.; Casida, J.E. *J. Neurochem.* **1985**, *45*, 798-804.
47. Lawrence, L.J.; Gee, K.W.; Yamamura, H.I. In *Benzodiazepine/GABA Receptors and Chloride Channels: Structural and Functional Properties*; Olsen, R.W.; Venter, J.C., Eds.; *Receptor Biochemistry and Methodology*; Alan R. Liss: New York, **1986**, Vol. 5; 225-240.
48. Sigel, E.; Barnard, E.A. *J. Biol. Chem.* **1984**, *259*, 7219-7223.
49. Stephenson, F.A.; Casalotti, S.O.; Mamalaki, C.; Barnard, E.A. *J. Neurochem.* **1986**, *46*, 854-861.
50. Cole, L.M.; Lawrence, L.J.; Casida, J.E. *Life Sci.* **1984**, *35*, 1755-1762.
51. Hawkinson, J.E.; Goeldner, M.P.; Palmer, C.J.; Casida, J.E. *J. Receptor Res.* **1991**, *11*, 391-405.
52. Lewin, A.H.; de Costa, B.R.; Rice, K.C.; Skolnick, P. *Mol. Pharmacol.* **1989**, *35*, 189-194.
53. Tanaka, K.; Matsumura, F. In *Membrane Receptors and Enzymes as Targets of Insecticidal Action*; Clark, J.M.; Matsumura, F., Eds.; Plenum: New York, **1986**; 33-49.
54. Olsen, R.W.; Szamraj, O.; Miller, T. *J. Neurochem.* **1989**, *52*, 1311-1318.
55. Cohen, E.; Casida, J.E. *Pestic. Biochem. Physiol.* **1986**, *25*, 63-72.
56. Brown, M.C.S.; Lunt, G.C.; Stapleton, A. *Comp. Biochem. Physiol.* **1989**, *92C*, 9-13.
57. Ozoe, Y.; Eto, M.; Mochida, K.; Nakamura, T. *Pestic. Biochem. Physiol.* **1986**, *26*, 263-274.
58. Nicholson, R.A.; Palmer, C.J.; Casida, J.E. *Pestic. Sci.* **1988**, *24*, 183-185.
59. Kadous, A.A.; Ghiasuddin, S.M.; Matsumura, F.; Scott, J.G.; Tanaka, K. *Pestic. Biochem. Physiol.* **1983**, *19*, 157-166.
60. Bloomquist, J.R.; Roush, R.T.; ffrench-Constant, R.H. *Arch. Insect Biochem. Physiol.* **1992**, *19*, 17-25.

61. Anthony, N.M.; Benner, E.A.; Rauh, J.J.; Sattelle, D.B. *Pestic. Sci.* **1991**, *33*, 223-230.
62. Bonner, J.C.; Yarbrough, J.D. *Pestic. Biochem. Physiol.* **1987**, *29*, 260-265.
63. *Ivermectin and Abamectin*; Campbell, W.C., Ed.; Springer-Verlag: New York, NY, **1989**.
64. Robertson, B. *Br. J. Pharmacol.* **1989**, *98*, 167-176.
65. Sigel, E.; Baur, R. *Mol. Pharmacol.* **1987**, *32*, 749-752.
66. Yamazaki, J.; Matsumoto, K.; Ono, H.; Fukuda, H. *Comp. Biochem. Physiol.* **1989**, *93C*, 97-104.
67. Duce, I.R.; Scott, R.H. *Br. J. Pharmacol.* **1985**, *85*, 395-401.
68. Scott, R.H.; Duce, I.R. *Pestic. Sci.* **1985**, *16*, 599-604.
69. Zufall, F.; Franke, C.; Hatt, H. *J. Exp. Biol.* **1989**, *142*, 191-205.
70. Soderland, D.M.; Adams, P.M.; Bloomquist, J.R. *Biochem. Biophys. Res. Comm.* **1987**, *146*, 692-698.
71. Holden-Dye, L.; Walker, R.J. *Parasitology*, **1990**, *101*, 265-271.
72. Martin, R.J.; Pennington, A.J. *Br. J. Pharmacol.* **1989**, *98*, 747-756.
73. Payne, G.T.; Soderlund, D.M. *J. Biochem. Toxicol.* **1991**, *6*, 283-292.
74. Lees, G.; Beadle, D.J. *Brain Res.* **1986**, *366*, 369-372.
75. Tanaka, K.; Matsumura, F. *Pestic. Biochem. Physiol.* **1985**, *24*, 124-135.
76. Arena, J.P.; Liu, K.K.; Paress, P.S.; Cully, D.F. *Mol. Pharmacol.* **1991**, *40*, 368-374.
77. Abalis, I.M.; Eldefrawi, A.T. *Pestic. Biochem. Physiol.* **1986**, *25*, 279-287.
78. Lummis, S.C.R.; Sattelle, D.B. *Pestic. Sci.* **1985**, *16*, 695-703.
79. Drexler, G.; Sieghart, W. *Eur. J. Pharmacol.* **1984**, *101*, 201-207.
80. Olsen, R.W.; Snowman, A.M. *J. Neurochem.* **1985**, *44*, 1074-1082.
81. Paul, S.M.; Skolnick, P.; Zatz, M. *Biochem. Biophys. Res. Comm.* **1980**, *96*, 632-638.
82. Pong, S.-S.; Wang, C.C. *J. Neurochem.* **1982**, *38*, 375-379.
83. Pong, S.-S.; DeHaven, R.; Wang, C.C. *J. Neurosci.* **1982**, *2*, 966-971.
84. Supavilai, P.; Karobath, M. *J. Neurochem.* **1981**, *36*, 798-803.
85. Pong, S.-S.; Wang, C.C. *Neuropharmacol.* **1980**, *19*, 311-317.
86. Drexler, G.; Sieghart, W. *Eur. J. Pharmacol.* **1984**, *99*, 269-277.
87. Schaeffer, J.M.; Haines, H.W. *Biochem. Pharmacol.* **1989**, *38*, 2329-2338.
88. Cully, D.F.; Paress, P.S. *Mol. Pharmacol.* **1991**, *40*, 326-332.
89. Drexler, G.; Sieghart, W. *Neurosci. Lett.* **1984**, *50*, 273-277.
90. Konno, Y.; Scott, J.G. *Pestic. Biochem. Physiol.* **1991**, *41*, 21-28.

RECEIVED July 29, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Chapter 11

Insect Growth Regulators for Pest Control, with Emphasis on Juvenile Hormone Analogs

Present Status and Future Prospects

J. Miyamoto, M. Hirano, Y. Takimoto, and M. Hatakoshi

**Sumitomo Chemical Company Ltd., Takarazuka Research Center, 4–2–1,
Takatsukasa, Takarazuka, Hyogo 665, Japan**

Two major groups of insect growth regulators (IGRs) are presently envisaged for possible pest control agents, chitin synthesis inhibitors (CSIs) and juvenile hormone analogs (JHAs). Certain CSIs are effective at 25 g a.i./ha against Lepidopteran larvae among others, developing molt disruption by inhibiting membrane transport of UDP-N-acetylglucosamine. However, CSIs may adversely affect non-target organisms including benificial insect species and crustaceans with chitin as a component of exoskeleton. Among JHAs, fenoxycarb is effective against Lepidoptera at 125 g a.i./ha, and pyriproxyfen is active against Diptera at 0.05 ppm in water and also affects reproduction of cladocera. JHAs interfere withnormal metamorphosis of insects by activating a certain gene(s) at inappropriate stages. The vertebrates and invertebrates except arthropoda appear considerably less susceptible to IGRs due to their intrinsic mode of action, and one thus expects reduced environmental deterioration. However, a wide range of precise information is required with regard to effects on a variety of arthropods for the exact assessment of their environmental impact.

Insect growth regulators (IGRs) have a unique mode of action; most of the conventional insecticides, *e.g.* organophosphates, carbamates and pyrethroids, are neurotoxins, but IGRs have an effect on insect-specific phenomena, so that selectivity between insects and mammals is expected. More importantly, potent IGRs are expected to be among the chemicals included in integrated pest management (IPM) to avoid or delay development of insecticide resistance.

IGRs are divided mainly into two groups by their mode of action; chitin synthesis inhibitors (CSIs) which disrupt molting of insects, and juvenile hormone analogs (JHAs) which interfere with metamorphic changes. Some of them are presently being used to control pest species in many fields.

0097–6156/93/0524–0144$07.25/0

In this paper characteristic efficacies, modes of action, and future perspectives of IGRs are discussed.

Chitin Synthesis Inhibitors

In the course of herbicide dichlobenil derivatives screening, DU-19111 (Figure 1) was found to have insecticidal activity. Its effect on insects was

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

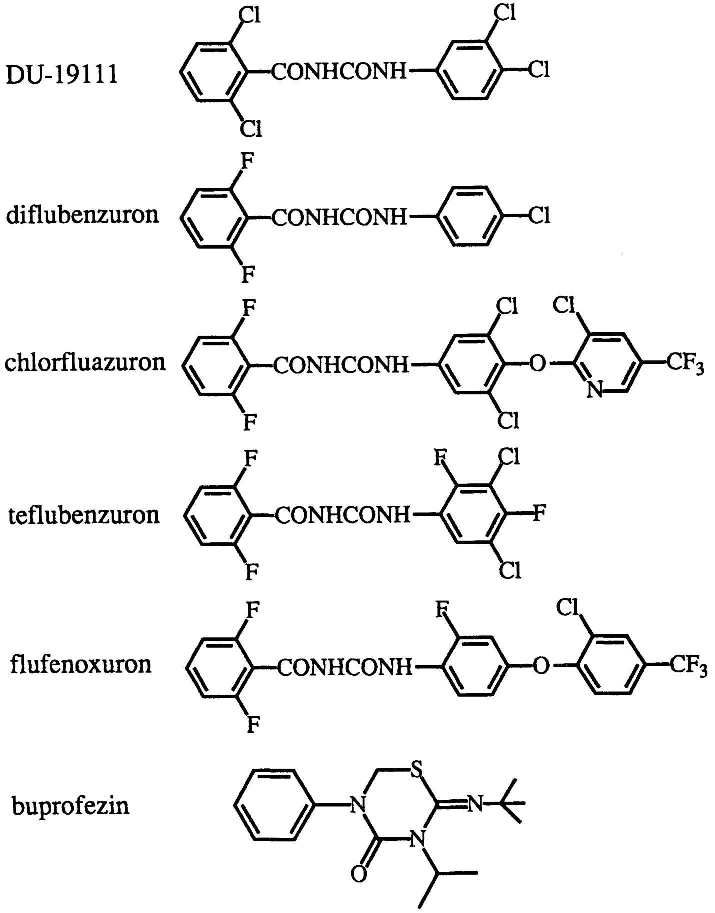

DU-19111
Cl
CONHCONH
Cl
Cl
Cl
diflubenzuron
F
CONHCONH
Cl
F
chlorfluazuron
F
Cl
Cl
CONHCONH
O
N
CF3
F
Cl
teflubenzuron
F
F
Cl
CONHCONH
F
F
Cl
flufenoxuron
F
F
Cl
CONHCONH
O
CF3
F
buprofezin
S
N
N
N
O

Fig. 1 Chemical structures of some chitin synthesis inhibitors.

found to be quite different from that of conventional insecticides, and death was thought to be connected with the molting process (*1*).

Diflubenzuron was selected as a more potent compound and this compound, the first CSI introduced into the market, inhibits chitin synthesis which is essential for exoskeleton formation.

Table I. Activity of Chitin Synthesis Inhibitor

Common Name	Activity Against *Spodoptera litura* LC50 (ppm)	Application Rate *Pectinophora sp.* (g ai/ha)
DU-19111	35	
Diflubenzuron	4.4	not effective
Chlorfluazuron	0.34	50-200
Teflubenzuron	0.12	50-62.5
Flufenoxuron	0.22	50-100

Against larvae of the common cutworm *Spodoptera litura*, diflubenzuron was eight times as active as DU-19111 (Table I). By structure modification of diflubenzuron, more active chlorfluazuron, teflubenzuron, and flufenoxuron were synthesized (Figure 1). These newer compounds are ten times as active as diflubenzuron against tobacco cutworm *Spodoptera litura* (Table I). The thiadiazin derivative buprofezin was also found to have chitin synthesis inhibitory activity (*2*).

Insecticidal activity. Benzoylphenyl urea derivatives are mainly used as larvicides. They control insect pests by disruption of normal molting, but ovicidal and sterilizing (reduction of viable eggs) activities are also demonstrated. Insect larvae molt at every instar, so that the sensitive periods of insects to CSIs encompasses all larval instars.

Diflubenzuron is effective against Lepidoptera, Coleoptera, and Diptera including flies and mosquitoes (*4*). It is used for forest, fruit and field crop protection. Application rates of diflubenzuron are about 50-75 g a.i./ha in forestry, 100-300 ppm in fruit growing, and 150 g a.i./ha in field crops. Chlorfluazuron is effective against many species of Lepidopteran insects in cotton, vegetables, and various insects in tea, maize, and other crops, but not against aphids, hoppers, and whiteflies (*5*). Teflubenzuron is used mainly against Lepidptera, Coleoptera, and Diptera. Different from other benzoylphenyl ureas, flufenoxuron is effective against mites in addition to Lepidopteran insects. Against the predatory mite *Amblyseius andersoni* on apples, this compound shows a comparable effect to fenbutatin oxide that is regarded as relatively harmless to the predator (Table II).

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Table II. Effect of Flufenoxuron on Predatory Mite, *Amblyseius stipulatus* on Citrus

Treatment	g ai/hl	No. of *A. stipulatus* /Leaf				
		1DBT	7DAT	10DAT	20DAT	32DAT
Flufenoxuron	10	3.9	3.2	2.4	2.3	1.9
	20	3.0	2.0	1.6	1.6	1.4
Fenbutatin oxide	44	3.3	2.0	3.3	2.5	0.9
Untreated	-	3.6	3.3	2.8	2.8	1.9

DBT: Days before treatment
DAT: Days after treatment

Another type of CSI, buprofezin is effective against Homopteran pests such as planthoppers, leafhoppers, whiteflies, and fruit scales (*6*). Similar to benzoylphenyl ureas, buprofezin does not kill adults, but reduces egg production and hatchability of the eggs from the treated adults, as shown in Table III.

Table III. Effect of Buprofezin on Adults and Progeny Formation of *Bemisia tabaci* under Greenhouse Conditions

Spray Concentration (ppm)	Adult Mortality (%)	Oviposition (eggs/female)	Egg Hatch (%)
500	0	6.3	-
250	0	5.8	-
125	0	5.3	13.2
62.5	0	5.8	14.2
31.25	0	7.4	28.9
Untreated	0	5.9	65.9

Mode of Action. When a chitin synthesis inhibitor is applied to insect larvae, they develop until molting (*3*), but fail in ecdysis due to inhibition of new cuticle synthesis. Biochemical studies show that these effects are induced by the inhibition of chitin biosynthesis. By direct action on the *Manduca* epidermal cells *in vitro*, diflubenzuron was shown to inhibit endocuticular deposition (*7*). Futhermore, UDP-N-acetylglucosamine was accumulated in the presence of diflubenzuron when 14C-glucosamine or 14C-acetylglucosamine was applied to final instar larvae of the cabbage armyworm *Mamestra brassicae* (*8,9*). The precursors of chitin, 14C-glucose in *Pieris* larvae (*10,11*), 14C-glucosamine in tobacco hornworm *Manduca sexta* larvae (*7*), 14C-acetylglucosamine in cabbage armyworm *Mamestra brassicae* larvae (*9*), or 14C-UDP-N-acetylglucosamine in cotton leafworm *Spodoptera littoralis* larvae (*12*) were not incorporated into chitin in the presence of CSIs *(13,14)*. Furthermore, the content of chitin was decreased.

American Chemical Society Library
1155 16th St. N. W.
Washington, D. C. 20036

However, cuticle protein content relative to fresh cuticle did not change (*13,14*).

The precise mode of action of diflubenzuron and other types of CSIs is still unknown; the information in the literature is fragmentary, and sometimes discrepant. As a hypothetical target site, at least three sites are considered: inhibitory action on chitin synthetase and/or inhibition of biosynthesis of chitin synthetase, inhibitory action on proteolytic enzyme and/or inhibition of biosynthesis of proteolytic enzyme and inhibition of UDP-N-acetylglucosamine transport through the membrane (Figure 2).

Diflubenzuron does not directly inhibit chitin synthetase from UDP-N-acetylglucosamine in cell-free preparations from pupae of the stable fly *Stomoxys calcitrans* (*15*), the last instar larvae of the cabbage armyworm *Mamestra brassicae* L. (*8*), the larval integument of the cabbage looper *Trichoplusia ni*, and pupae of the cecropia moth *Hyalophora cecropia* (*16*). However, antibiotics such as polyoxin and/or nikkomycin do the chitin inhibit synthetase (*8,16*).

The inhibition of proteolytic enzyme, which activates chitin synthetase zymogen, was reported (*17*). Diflubenzuron was shown to inhibit the activity of proteolytic enzymes such as chymotrypsin and trypsin (*18*). Both experimental systems, however, were derived from the fungus *Phycomyces* and no experiment was carried out using insect preparations.

When the midgut of the cabbage armyworm *Mamestra brassicae* was turned inside out, the inhibition of transportation of UDP-N-acetylglucosamine through the membrane was suggested to be the primary action of diflubenzuron (*9*). In that experiment, the midgut from last instar larvae was cultured with 14C-N-acetylglucosamine. Diflubenzuron induced accumulation of 14C-UDP-N-acetylglucosamine about 1.7 times as much as in the control midgut when 14C-acetylglucosamine was applied in the medium and inhibited chitin synthesis almost completely. When 14C-acetylglucosamine was applied inside of the midgut which had been turned inside out, with or without diflubenzuron in the medium, the sum of 14C-UDP-N-acetylglucosamine in the medium and 14C-chitin in the outside of the diflubenzuron-treated midguts were reduced to about 1/4 of the control. 14C-UDP-N-acetylglucosamine in the diflubenzuron-treated tissue, however, accumulated 1.6 times as much as those in the control, although this result should be confirmed. The chitin synthesis was not inhibited by diflubenzuron present in the outer medium, when UDP-N-acetylglucosamine was applied directly to the face of cuticle-producing membrane. These results show that UDP-N-acetylglucosamine accumulated in the cells with less leakage from the midgut cells and less amount of cuticle formation when diflubenzuron was applied. And therefore, they lead to the conclusion that diflubenzuron seems to inhibit the transportation of UDP-N-acetylglucosamine through the biomembrane.

Juvenile Hormone Analogs

Five known insect juvenile hormones (JH) (Figure 3) are synthesized and secreted from the corpora allata (CA). As a physiological role, the JHs possess a *status quo* effect during larval development. Furthermore, in some species of insects JHs control ovarian development, larval and adult diapause, and phase-variation. After JH had been known to be an insect-specific material, Williams (*19,20*) proposed that it would become an insect-

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

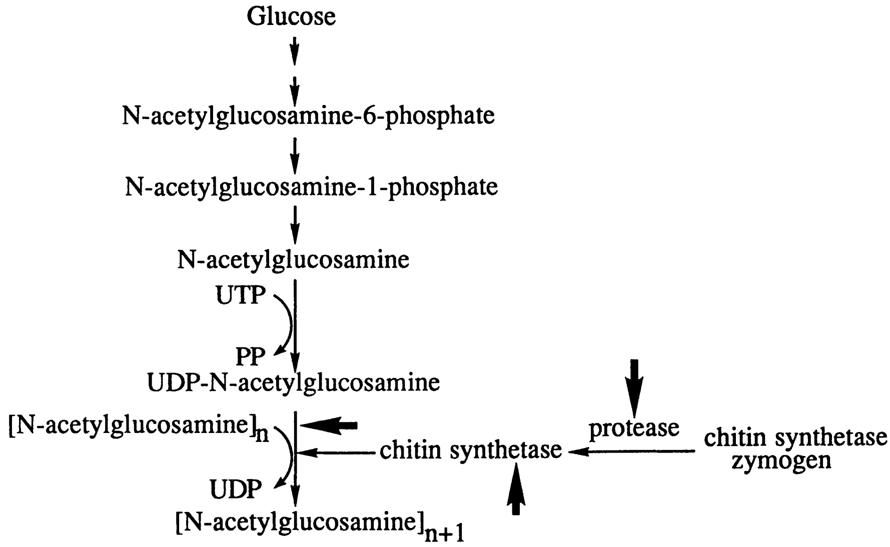

Glucose
N-acetylglucosamine-6-phosphate
N-acetylglucosamine-1-phosphate
N-acetylglucosamine
UTP
PP
UDP-N-acetylglucosamine
[N-acetylglucosamine]$_n$
UDP
[N-acetylglucosamine]$_{n+1}$
chitin synthetase
protease
chitin synthetase zymogen

Fig. 2 Biosynthetic pathway of chitin. Each bold arrow indicates a hypothetical target site of chitin synthesis inhibitors.

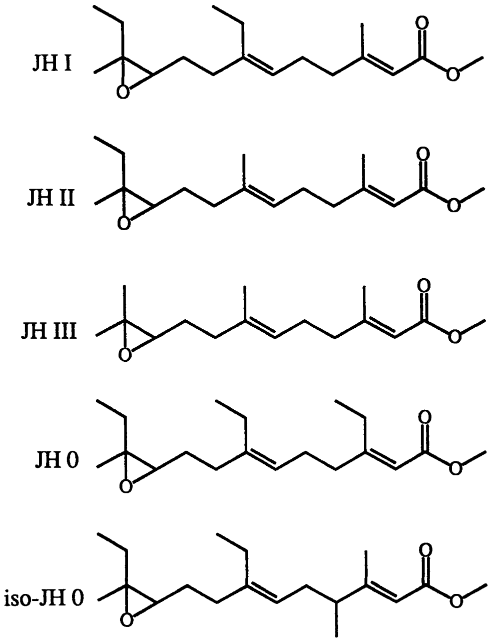

JH I
O
O
JH II
O
O
JH III
O
O
JH 0
O
O
iso-JH 0
O
O

Fig. 3 Structure of natural JHs.

specific control agent. But their stability and synthetic difficulties did not allow the use of JH itself for pest control. However, many analogs (JHA) have been synthesized and tested for their activities against many species of insects (*21*). Terpenoid compounds such as methoprene (Figure 4) were first introduced in the market, but their use had been restricted to household pests, due to lack of high activity against agricultural pests and low residues on plants under field conditions. Fenoxycarb was the first compound used against agricultural pests, followed by pyriproxyfen (Figure 4).

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Insecticidal Activity. As mentioned above, JHAs affect morphogenesis, reproduction, embryogenesis, and also diapause and phase variations. JHAs control insect pests mainly by morphogenetic, reproductive (sterile), and embryogenetic effects. Only the early phase of the last larval instar is the main sensitive stage, which differs from CSIs. The main effects contributing to insect control are dependent on target pests. Methoprene, which induces morphogenetic effects at pupation or adult emergence without affecting larval development in mosquitoes, as well as death at higher concentrations, is effective against insect pests of public health and veterinary importance and stored product pests. Fenoxycarb is shown to be effective against cockroaches, fleas, and ants, and is also marketed for use against summer fruit tortrix moths, fruit tree tortrix moths, codling moths, other moths attacking fruit trees and vines, leaf rollers, pear psyllids, and scales (4). Fenoxycarb, furthermore, is suggested to be active against stored product pests such as the lesser grain borer, flour beetle, and Indian meal moth. Pyriproxyfen was registered in Japan in 1991 for pest control in public health. This compound was shown to be active against many species of mosquitoes and the housefly (*22-25*). The efficacy was confirmed in the field against the mosquito *Anopheles farauti* (*26*) and the housefly *Musca domestica* (*27*). Pyriproxyfen is reported to be active against green peach aphid *Myzus persicae*, arrowhead scale *Unaspis yanonensis* (*28*), greenhouse whitefly *Trialeurodes vaporariorum* (*29*), tea scale *Fiorinia theae* (*30*), and pear psylla *Psylla pyricola* (*31*). Tables IV and V show the efficacy of pyriproxyfen against pear psylla and its predators (*31*). These results show a high efficacy of pyriproxyfen at 125 g a.i./ha and no adverse effects on predators at the same dosage.

Table IV. Effect of Pyriproxyfen on Pear Psylla, *Psylla pyricola*

Treatment	Rate (g ai/ha)	Mean No. of Nymphs/50 Leaves			
		9DBT	8DAT	35DAT	49DAT
Pyriproxyfen	125	42.0	13.8	2.3	1.3
	63	48.0	40.8	36.0	54.0
Untreated	-	55.0	61.8	89.5	124.3

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

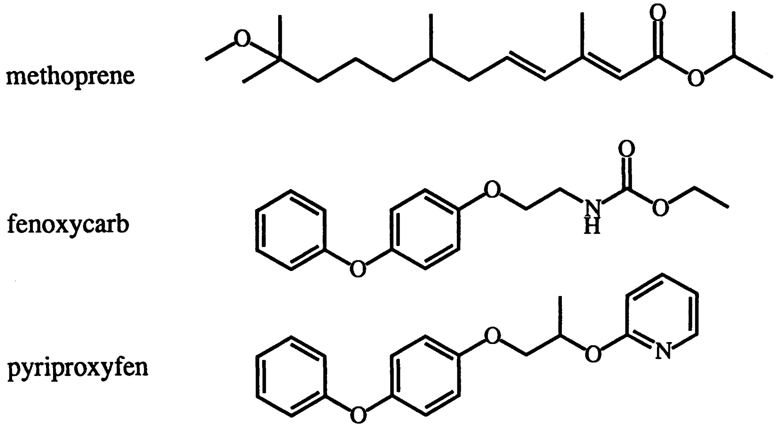

methoprene
O
O
O
fenoxycarb
O
O
N
H
O
O
pyriproxyfen
O
O
O
N

Fig. 4 Structure of some JHAs.

Table V. Effect of JHA on Predators[a] of Pear Psylla

Treatment	Rate (g ai/ha)	Mean No. of Predators/Sample[b] 6DBT	4DAT	21DAT	48DAT	62DAT
Pyriproxyfen	125	10.7	8.4	6.7	4.8	5.1
Fenoxycarb	125	13.7	11.0	8.6	4.8	4.9
Untreated	-	11.8	12.8	7.4	4.6	5.8

[a] *Anthocoris antevolens*
A. nemoralis
Deraeocoris brevis
Campylomma verbasci
Chrysopa spp.

[b] Four limbs from each of the four trees were sampled (n=16).

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Mode of Action. When JHA is applied to the last instar Lepidopteran larvae, it induces various abnormalities after molting. Significantly, this effect depends on the timing of application. This reflects the hormonal milieu in the insects; in the last instar larvae, JH disappears just after or within a few days after final molting to larvae. In tobacco hornworm *Manduca sexta* the titer of hemolymph JH begins to decline on day 2 (*32*). In this insect, prothoracicotropic hormone is released on day 3 to stimulate the prothoracic glands to secrete small amount of ecdysone. This small surge of ecdysteroid without JH induces commitment from larval development to pupal development (*33*), suggesting that application of JHAs after pupal commitment has no effect on the morphological changes. Thus, the sensitive period of the last instar larvae is between disappearance of JH and before appearance of the small surge of ecdysteroid. When pyriproxyfen is applied at high doses to last instar larvae of tobacco cutworm *Spodoptera litura* (100 μg) or tobbaco hornworm *Manduca sexta* (10 μg) after a decrease of JH, the treated larvae molted into supernumerary larvae (*34,35*). In these larvae, the brain is presumed to be activated to secrete prothoracicotropic hormone by the presence of a high concentration of pyriproxyfen for an adequate period. Ecdysteroid titer peaks in penultimate instar larvae, and pyriproxyfen seems to induce larval molt. JHAs are in general hydrophobic compounds, so that they must bind to a certain extent to carrier proteins in the hemolymph; JH also binds to JH binding proteins to avoid enzymatic metabolism and non-specific adsorption to various tissues (*36*). Actually JHs are removed from hemolymph by JH specific esterase and cessation of JH production. One of the JH binding proteins from tobacco hornworm *Manduca sexta* was purified and characterized; molecular weight as 28 kDa, one binding site per molecule, $Ka=1X10^7M^{-1}$, and pI=5.0. This protein does not have a capacity to bind JHA methoprene and JH metabolites (*36*). JH or JHA bound to the carrier protein will be transported to the target cells, binding to a cytosolic or nuclear protein after entering the cell. *Manduca* cytosolic binding proteins have 38 kDa in molecular weight. In the nuclei, JH and JHA bind to receptor proteins whose molecular weights are 29 kDa, with

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

isoelectric point of 5.8 for JH and 6.0 for JHA. The 29 kDa nuclear JH binding protein, a putative JH receptor, from the epidermis of *Manduca sexta* was shown to bind DNA cellulose, and furthermore, the protein binds to endocuticle genes which are regulated by JH and code the larval cuticle proteins (*37,38*). However the identity of the JH binding protein and nuclear protein has not yet been confirmed. One of the genes which encode a larval cuticle protein has two regions which bind to the nuclear protein; a 250 bp region (-241 to -491) 5' to the gene and a 200 bp region in the first intron (+209 to +409) (*39*).

Effect of IGRs on Non-target Organisms

As mentioned above IGRs differ profoundly in their mode of action from conventional neurotoxic insecticides. Therefore, the IGRs might exert a characteristic environmental impact differing from the foregoing insecticides.

From the viewpoint of reduced environmental impacts of IGRs, certain characteristics of ecological effects of IGRs will be briefly summarized with specific reference to the effect of pyriproxyfen on aquatic environments. For additional information on environmental impacts of IGRs, refer to review articles cited (*40-43*).

Ecological Effects of a Chitin Synthesis Inhibitor, Diflubenzuron. Chitin is selectively distributed among living organisms. It is a common cell wall constituent in fungi and green algae and absent in the Tracheophta. In animals, chitin occurs in most invertebrate phyla including arthropods, but is absent among vertebrates.

Since arthropods share the same molting process, the species-specificity of CSIs might be less pronounced than JHAs, although obviously the selectivity is apparently related to the ingestion of residues as well as metabolism.

Aquatic Environment. Diflubenzuron at its actual application rates produces no adverse effects on Chlorophyta, Chrysophyta, Rotifera, Platyhelminthes, Nematoda, Mollusca, Annelida, Arachnida, Amphibia, and Pisces. Although most rotifers have a chitinous lorica around their bodies, application of diflubenzuron at 2.5-10 ppb did not affect the abundance of rotifers (*44*). The affected species are in the Arthropoda; some crustaceans such as waterfleas, *Daphnia* and *Moina* sp., tadpole shrimp, *Triops* sp., and clam shrimp, *Eulimnadia* sp., were extremely sensitive, showing LC50 values about 0.1-1 ppb, being comparable to mosquito LC50 values of *ca.* 0.7 ppb. Copepods such as *Cyclops* and *Diaptomus* sp. are somewhat tolerant, with LC50 values ranging from 0.01 to 0.05 ppm. Among insects, mayfly nymphs and chiromid larvae were relatively sensitive, whereas aquatic beetles showed a strong tolerance (*45,46*).

With respect to the effects on macrocrustacea, diflubenzuron at ppb levels affects survival (*47*), development of larvae (*48*), regeneration (*49*), and reproduction (*47*). Mortality was associated with inability to cast off the exuviae at ecdysis.

Application of diflubenzuron to waterbodies resulted in population reduction of several crustaceans of Amphipoda, Cladocera and Copepoda, *Bosmina longirostris, Daphnia pulex, D. galeata, Diaptomus* sp., *Hyalella*

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

azteca, *Cyclops* sp., and *Cyprinotus* sp. (*44,50,51*), as well as aquatic insects of hydrophilid beetles and naiads of libellulid dragonflies (*52*). Adult and immature notonectids and corixids as well as adult *Thermonectus* sp. were not affected. One to three weeks were needed for microcrustaceans to resurge after treatment. Several studies show that diflubenzuron affects the abundance of microcrustaceans and the recovery time for these animals ranges from one week to as long as three months, depending on species, rate of application and width of treated area.

Although the growth and survival of bluegill sunfish *Lepomis macrochirus* were not affected in the lake receiving initial diflubenzuron residues of 3.3 ppb, this fish showed a shift from feeding on cladocerans and copepods to chironomid midges and terrestrial insects, due to elimination of cladocerans, apparently being more susceptible to the IGR than other arthropods (*44*).

Terrestrial Environment. *Trichogramma pretiosum*, *Apanteles maginventris* and *Voria ruralis* were not affected when treated as adults on cotton fields. Rates of parasitism after treatment as well as the survival of the F1 generation were found to be unaffected (*53*). Among a number of predators of boll weevils, only green lacewing *Chrysopa carnea* and convergent lady beetle *Hippodamia convergens* showed significant decreases in egg hatching due to diflubenzuron treatments (*54*). *Geocoris punctipes* nymphs were adversely affected (*55*). Effects on other predators and parasites during field treatment tend to be marginal, and the reduction appeared to be minor compared with the effects of conventional insecticides.

Bees. After treatment of alfalfa with diflubenzuron, negligible mortality to adult honeybees, leafcutting bees, and alkali bees were observed 48 hr after residual treatments (*56*). Diflubenzuron sprayed to control the sugarcane rootstock borer weevil *Diaprepes abbreviatus* had no effect on honeybee brood of resident colonies in the sprayed area over a 7-month period (*57*).

Ecological Effects of a JHA, Methoprene. The insect-specific mechanisms demonstrated by JHAs during metamorphosis are 1) embryocidal at pre- and/or post-oviposition, 2) morphogenetic damage at metamorphosis and caste differentiation, and 3) sterilization to male and/or female (*58*). JHAs have been synthesized to resemble the natural JH 0-III of insects in their configurations. Methyl farnesoate, the unepoxidized form of JH III is speculated to be a juvenile hormone of crustaceans (*59*). Farnesoic acid and its methyl ester have been detected in the Decapoda among the crustacea such as the brachyurans of the mud crab *Scylla serrata*, the spider crab *Libina emarginata*, *Cancer borealis* and *C. maenas*, and the macurans of the crayfish, *Procambarus clarkii*, and the lobster, *Homarus americanus* (*60,61*).

The Decapoda produce farnesoic acid (FA) and its methyl ester (MF) from the mandibular organ, homologous to the insect corpora allata (CA), but are not able to epoxidize them to JH and JH acid. Insects have the epoxidase(s) to generate JH acid and JH from FA and MF. Among the insects, two highly evolved orders of the Lepidoptera and the Diptera are proposed to produce the more complex forms of JH, including JH 0, JH I, 4-

methyl JH I, JH II, and JHB3 (bisepoxide JH III). The evolution of more complex molecules and the simultaneous occurrence of two or more of these substances in one insect may reflect the requirement for different JHs to assume the regulation of different physiological events in the more evolved taxa (*61*). This hypothesis might suggest wide species-specificities among the target as well as nontarget organisms in the arthropods. To produce more species-specific insecticides and fully evaluate effects on nontarget organisms, more effort should be put into the identification of specific biological roles for each of these products across all arthropod groups.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Aquatic Environment. The acute toxicities of methoprene to ten aquatic organisms show the least tolerant is *Daphnia magna* (24 hr LC50=0.9 ppm), and the most tolerant is tadpole shrimp, *Triops longicaudatus,* (96 hr LC50=5.0 ppm). Among 30 species including Protozoa (1 species), Platyhelminthes (1 species), Rotatoria (1 species), Annelida (3 species), Arthropoda (6 Crustacea and 14 Insecta), Mollusca (2 species), and Chordata (2 species), most organisms tolerate up to 1 ppm dosage level except aquatic Diptera, *Brachydeutera argentata* (shorefly), *Chironomus stigmaterus* (midge), and *Pericoma* sp. (mothfly). When late-instar larvae of these Diptera were exposed to dosages of 0.01 to 0.1 ppm, 50% or more pupae were unable to emerge. Exposure to 5 species of algae at 0.1 ppm for a week resulted in no adverse effect (*62*).

The 1st zoeal stage of mud crab *Rhithropanopeus harrisii* appeared to be the most sensitive of all larval stages to methoprene. The duration of larval development was significantly prolonged with increasing concentrations of methoprene (0.01-1 ppm). However, methoprene did not inhibit the metamorphosis of this crab at 0.1 ppm or less (*63*).

Methoprene at field rates, like CSIs, produces no adverse effects on Rotifera, Platyhelminthes, Nematoda, Mollusca, Arachnida, and Pisces. Furthermore, no adverse effects are observed in field studies on Cladocera, *Daphnia* and *Moina* sp., Eucopepoda, *Cyclops* and *Diaptoms* sp., Conchostraca, *Eulimnadia* sp., and Podocopa, *Cypricerus* sp. and the ostracod, *Cyprinotus* sp. (*64,65*).

In some cases, methoprene produces short-term toxic effects on the waterflea *Daphnia magna,* Copepod *Cyclop* sp., side-swimmer *Hyalella azteca,* clam shrimp *Eulimnadia* sp., seed shrimp *Cypricercus* sp., and tadpole shrimp *Triops longicaudatus* (*62*) and on copepod populations inhabiting salt marsh habitats (*66*).

These studies reveal that field applications of methoprene may cause temporary stress on some microcrustaceans. However, it offers considerable safety to microcrustaceans without producing any long-term disruptions in the population levels of these animals.

Multiple applications of methoprene at 302 g a.i./ha to experimental ponds significantly affected populations of mayfly *Callibaetis pacificus* and certain aquatic coleopterans, *Laccophilus* sp. and *Tropisternus lateralis.* In these habitats, naiads of anisopterans, *Anax junius*, *Erythemis simplicicolis,* and *Tarnetrum corruptum* and the zygopteran, *Enallagma civile* were not affected by methoprene treatments (*65*).

These findings on JHAs reveal that JHAs clearly have species- and stage-specificities to nontarget organisms.

Terrestrial Environment. With respect to predators, the carmine spider mite *Tetranychus cinnabarinus* tolerates a high dose of JHAs. The tarnished plant bug *Lygus lineolaris* and cotton aphid showed characteristic hormone effects. Of the predators, the lace-wing, *Chrysopa carnea* and the predacious lygaeid bug, *Geocoris punctipes* tolerated higher doses than their insect pest counterparts. The convergent lady bug, *Hippodamia convergens*, however, was sensitive to many JHAs. Certain effects of JHAs have potential for practical application (*67*). The predacious mite, *Amblyseius brazilli*, was not affected by topical treatment as high as 1000 ppm of methoprene, but when fed with pollen at 100 ppm egg-laying was inhibited (*68*).

With respect to parasites, eggs of gypsy moth, *Porthetria dispar*, parasitized with *Ooencyrtus kuwanai* were treated with the JHAs of hydroprene and R-20458. The ED50 for gypsy moth eggs was 6.3 ng/egg, whereas the dose that produced deleterious effects on the parasites was 63 ng/egg. The larval parasite, *Apanteles melanoscelus*, was only marginally affected (*69,70*). Parasites, *Cardiochiles nigriceps* and *Canipaletis sonorensis* of the tabacco budworm *Heliothis virescens* were affected by treatment of a JHA. There was a delay in the emergence of the parasites and there was also a decrease in the number of the former braconid wasps that emerged (*71*). Hydroprene, triprene, and kinoprene were found to have adverse effects on the parasite, *Aphidius nigripes* of the potato aphid *Macrosiphum euphorbiae* (*72*). While JHAs do affect parasites, their adverse effects are less than those of broad-spectrum, conventional insecticides.

Honeybees. Age polyethism occurs in many highly eusocial bees, such as the honey bee (Apinae) and stingless bees (Meliponinae). Workers perform different ensembles of tasks as they age; young workers perform tasks such as brood and queen care and nest maintanance within the nest, and older individuals forage. Bumble bees (Bombinae) share a common origin with the Apinae and Meliponinae but are primitively eusocial and exhibit a different pattern of division of labor (*73*).

JH is involved in the regulation of age polyethism in honey bee *Apis mellifera*. JH titer increases with worker age, as does the volume of the CA, the glands that produce JH. Removal of the CA blocks the degeneration of the hypopharyngeal glands that produce brood food (*74*).

Topical application of 250 μg of methoprene to adult worker honey bees can cause a premature shift from the broodnest to food storage region, precocious foraging behaviour, and premature production of the two alarm pheromones. The worker longevity was proportional to the age at which foraging commenced; those treated with methoprene had a significantly shorter life span (*75*).

One of the strongest effects elicited by JHA treatments is the induction of premature foraging; by topical treatments at 2 μg hydroprene or 200 μg of methoprene and oral treatment at 20 μg hydroprene or 1 mg methoprene (*74*).

When honeybees were injected at 0.1-10 μg/bee or treated topically at 0.5-10 μg/bee of methoprene to day-0 workers, guard bees and pollen-foragers appeared 7 and 5 days earlier than the control, respectively, by inhibition of the development of CA due to negative-feedback (*76*).

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

When colonies were fed 1000 ppm of JHA in honey, worker bees were unaffected but broods were affected. The worker bees removed poisoned larvae. These effects were less than that of conventional insecticides, such as mevinphos, parathion, and methylparathion (*77,78*).

Topical application of 250 µg of methoprene or injection of 250 µg JH I did not affect worker foraging or nest activity in colonies of the primitively eusocial bumble bee *Bombus impatiens* and *B. bimaculatus*. Because bumble bees do not exhibit strong age polyethism, these results are consistent with findings in honey bees (*73*).

Ecological Effects of a New JHA, Pyriproxyfen

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Mammalian toxicological profiles are not discussed here. Toxicities of pyriproxyfen to nontarget aquatic organisms are similar to those of methoprene; crustaceans and aquatic insect larvae are sensitive. During 21-day reproduction studies, *Daphnia pulex* and *D. magna* produced fewer numbers of young/adult. At the level which affected reproduction, the growth of both waterfleas was also affected, although the survival rates were not significantly different from the control and numbers of molting successfully occurred. Figure 5 shows that at higher concentration body length of *D. pulex* is shorter than that of the control and the waterflea has fewer embryos in the brood chamber. In order to examine the effects on recovery, *Daphnia pulex* were transferred to fresh water free from pyriproxyfen. The waterfleas produced sufficient numbers of young in one week as shown in Figure 6. Therefore, the effects are demonstrated to be reversible.

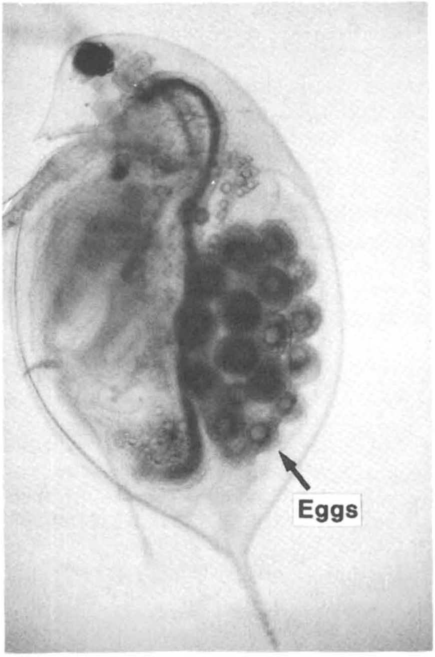
Last instar of aquatic insect larvae are most susceptible to pyriproxyfen like other JHAs. The susceptibility difference is more than 1000 times between the last instar and pre-last instar of dragonfly, *Orthetrum albistrum speciosum* and midge, *Chironomus yoshimatsui*. Even in the last instar of dragonfly larvae, the initial 8 days are the most susceptible of the 40-day period. Morphogenetic aberrations were observed in the form of emergence inhibition at the lower dosage and supernumerary at higher dosage.

Since pyriproxyfen has high activity against mosquito larvae with an EC95 (inhibition of emergence of 95%) at sub-ppb levels against 4th-instar mosquito larvae of *Anopheles quadrimaculatus*, *Aedes aegypti*, *Aedes taeniorhynchus*, *Culex tarsalis*, and *Culex quinquefaciatus* (*24,79*), several field studies have been carried out to assess its effects on other aquatic organisms.

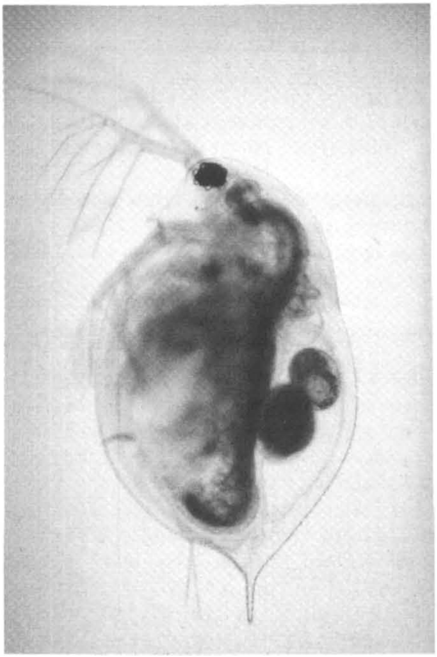
Pyriproxyfen applied at field rates of 5.6, 11.2, and 28.0 g a.i./ha did not exhibit any marked ill effects during 21-day on nontarget organisms prevailing in the experimental ponds; mayfly naiads, *Callibaetis pacificus*, dragonfly naiads, *Tarnetrum corruptom*, and *Anex jubius*, several species of diving beetle larvae and adults, Hydrophilidae, and Dytiscidae and two species of ostracods, *Cypridopsis* sp. and *Cyprinotus* sp. (*23*).

No significant effects were detected on mixed populations of cladocerans, *Simocephalus* sp. and *Alona* sp. and copepods, *Cyclops vernalis* which were treated with 0.01 ppm pyriproxyfen during a 2-week test period. Pyriproxyfen applied at the effective dosage of 0.0056 kg a.i./ha to two rice plots resulted in no adverse effects on a wide variety of aquatic nontarget organisms, cladocerans, *Simocephalus* sp. and *Alona* sp.;

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011



Control



Pyriproxyfen at 60 μg/L

Fig. 5 Effect of pyriproxyfen on reproduction of *Daphnia pulex.*

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

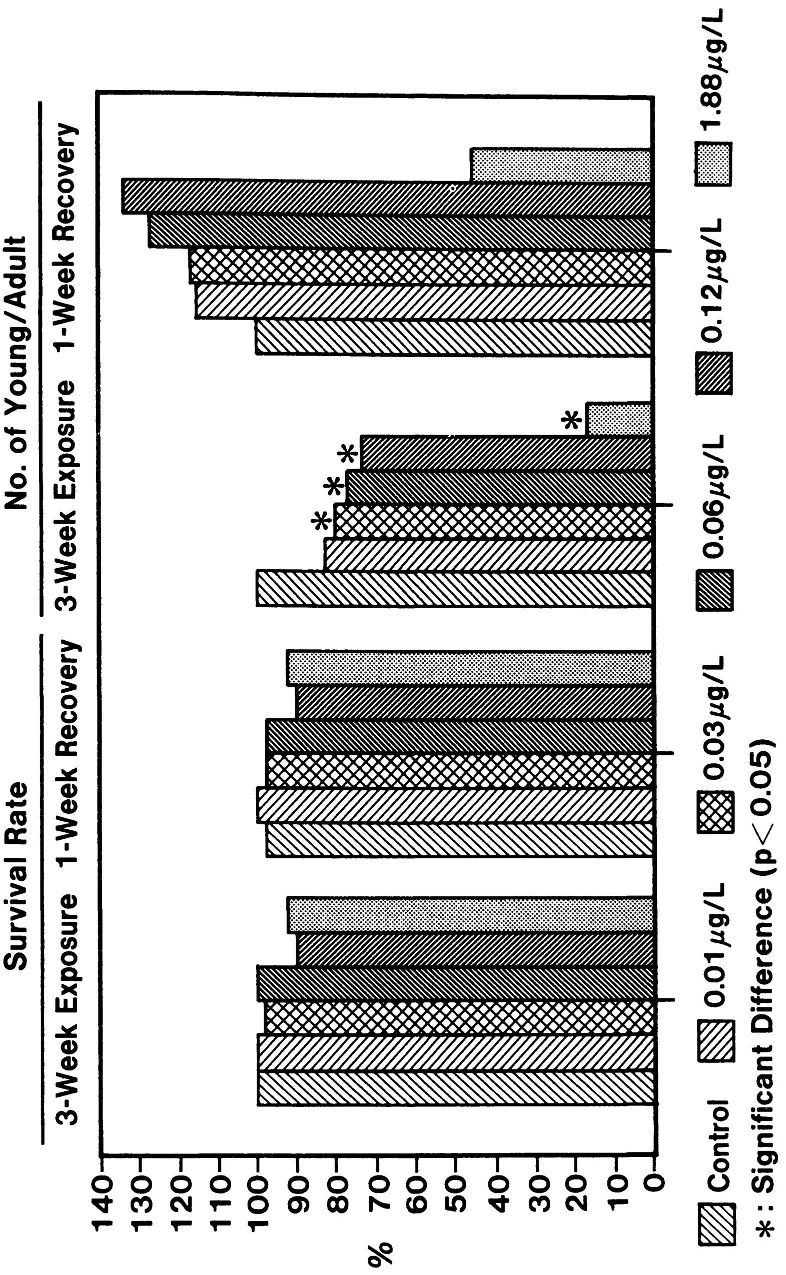


Fig. 6 Effects of pyriproxyfen on reproduction of *Daphnia pulex*.

copepods, *Cyclops vernalis*; ostracods, *Cypris* sp.; dragonflies, *Anax junius* and *Pantala hymenaea*; beetles, *Tropisternus* sp., *Hydrophilus triangularis*, *Laccophilus* sp. and *Copelatus* sp.; chironomids, *Chironomus stigmaterus* and *Goeldichironomus holoprasinus*; ceratopogonids, *Culicoides varipennis*; and hydra (*79*).

When pyriproxyfen was applied twice at high rates of 0.05 and 0.11 kg a.i./ha (8 and 20 times greater than the effective dosage, respectively) to experimental rice plots, minor suppression of the reproductive capacity of daphnoid cladocerans and ostracods were observed. A low degree of induction of morphogenetic aberrations in Odonata (Anisoptera and Zygoptera) at adult emergence was exhibited. The deformed adults were observed 4-10 days after treatment. Aquatic beetle adults, dragonfly nymphs, and lycosid spiders showed no adverse effects from the treatments (*80*).

Degradation in the Environment

Photodegradation in water. C-14 pyriproxyfen was dissolved in distilled water or sterile river water at the concentration of 0.2 ppm, and the solutions were exposed to natural sunlight from November to December at 40 degrees north Latitude. The half-lives of pyriproxyfen were found to be 17.5 and 21 days, respectively. Since the quantum yield of the compound is found to be 0.0866, the calculated half-lives at 40° latitude are 7.9, 4.4, 15.8, and 62 days at spring, summer, fall, and winter, respectively. Photodegradation pathways are cleavage at three ether linkages and the major products were CO2 and a dephenoxyphenyl product which account for 11-29% and 16-30% of the applied C14, respectively.

Photodegradation on Soil. C-14 pyriproxyfen was applied on sandy loam and silty loam soils and exposed to sunlight for 8 weeks. The half-lives of pyriproxyfen were found to be 11 and 13 weeks, respectively. Under dark conditions, more than 87% of the applied C14 was recovered as the parent compound. Photodegradation pathways were cleavage at three ether linkages. The major degradate was CO2 from the phenyl labeled pyriproxyfen, producing 9.5-13% of the applied C14. Sunlight exposure produced bound C14, amounting to 11-26% of the applied C14 at week 8.

Aerobic Soil Metabolism. C-14 pyriproxyfen was applied at a concentration of about 0.5 ppm to sandy clay soil and incubated at 25°C under dark condition for 30 days. The half-life was 6.3 days and the residue at 30 days was 0.12-0.13 ppm. The major degradation pathways were hydroxylation at 4'-position of the phenoxyphenyl ring and cleavage at three ether bonds, and the phenoxy ring and pyridyl ring were finally decomposed to CO2.

Degradation in Water/Sediment System. Two sandy loam soils (about 1.3 kg) were put on the bottom of each tank, 10 L of water was added, and the system was conditioned for 7-10 days at 25°C. C-14 pyriproxyfen in a granule formulation was applied at a concentration of 50 ppb a.i. and kept for 28 days. The maximum water concentrations eluted from the formulation were dependent on the organic matter contents in the soils, being 3 ppb at 6.3% organic matter and 7 ppb at 0.9% on days 3-5. Then, the concentrations decreased with time and half-lives were calculated to be

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

7-14 days. On day 28, distributions of C14 in water and soil were 8.3% and 80.0% in higher organic matter soil and 39.0% and 36.8% in other soil, respectively. Contents of pyriproxyfen in these soils on day 28 were 58% and 18% of the applied C14, respectively. The major degradation pathways were hydroxylation at 4'-position of the phenoxyphenyl ring and dephenylation.

From these laboratory studies, pyriproxyfen is decomposed abiotically and biotically through ether cleavage and hydroxylation. Degradation pathways and half-lives of pyriproxyfen are shown in Figure 7 and Table VI, respectively.

Table VI. Half-lives of Pyriproxyfen in Aquatic Environments

Study	Medium	Half-life
Photodegradation in water	Distilled/Sunlight	2.5 Weeks
	River/Sunlight	3 Weeks
Photodegradation in soil	Sandy loam/Sunlight	11 Weeks
	Silty loam/Sunlight	13 Weeks
Hydrolysis in water	Buffer (pH 5,7,9)	>5 Months
Aerobic soil metabolism	Sandy clay loam	6 Days
Water/sediment degradation	Sandy loam	7 Days
	Sandy loam	14 Days
Natural ponds	Water	1.6 Days
	Sediment	10 Days

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Decline in the Natural Ponds. Pyriproxyfen 0.5% formulation was applied to seven ponds at a concentration of 50 ppb a.i. The concentrations of pyriproxyfen in water were 2.6 ppb at its maximum and 1.7 ppb on the average on the first day of application. Then, the concentrations decreased rapidly, with mean half-life of 1.6 days. In the sediments, mean half-life was calculated to be 10 days.

Persistence of pyriproxyfen in water and soil of the treated pond was of short duration, where no residues were detected (<0.0002 ppm) 48 hr after treatment even at 0.045 kg a.i./ha (*79*).

Pyriproxyfen applied twice at high rates of 0.05 and 0.11 kg a.i./ha (8 and 20 times greater than the effective dosage, respectively) to experimental rice plots, resulted in no detectable residues in water (<0.05 ppb) in 2 days, in soil (<0.001 ppm) on any dates and in bluegill sunfish (<0.005 ppm) after 3 days (*80*).

When pyriproxyfen was applied to wastewater lagoon at 0.11 kg a.i./ha, no residues were detected in water (<0.04 ppb) from day 1 to day 64 after treatment. The concentrations in organic matter were 0.14 ppm on day 1 of treatment then decreased at an exponential rate with half-life of 7.5 days (*81*). Thus, pyriproxyfen is readily adsorbed onto organic matter and then the concentration in the aquatic compartments declined rapidly.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

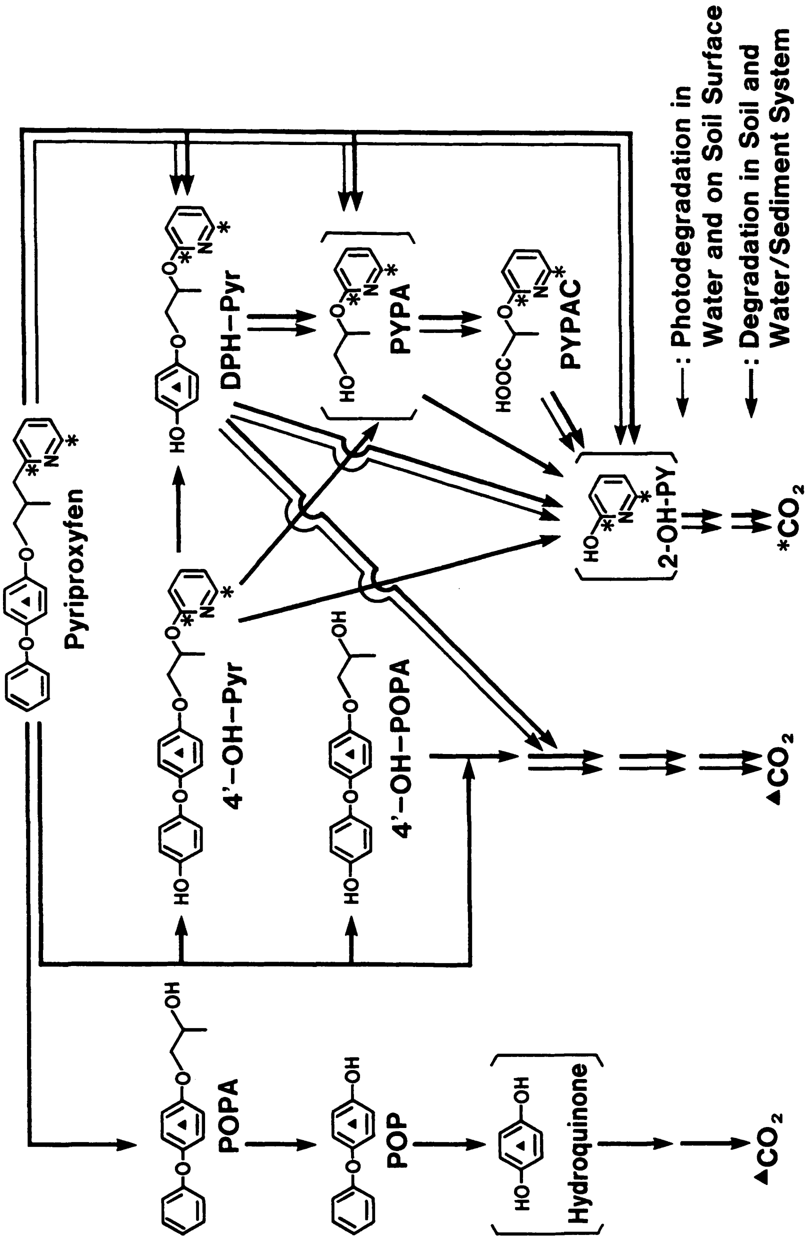

Pyriproxyfen
DPH-Pyr
PYPA
PYPAC
4'-OH-Pyr
4'-OH-POPA
2-OH-PY
$*CO_2$
$\blacktriangle CO_2$
POPA
POP
Hydroquinone
⟵ : Photodegradation in Water and on Soil Surface
⟵ : Degradation in Soil and Water/Sediment System

Fig. 7 Proposed degradation pathways of pyriproxyfen in water, soil and water/sediment system.

Bioaccumulation and Metabolism in Aquatic Organisms. When carp (*Cyrinus carpio*) were exposed to C-14 pyriproxyfen at a concentration of 2 ppb under flow-through system for 14 days, the concentration of C14 and pyriproxyfen in the fish body reached an equilibrium after a 3-day exposure. The bioaccumulation ratios for C14 and the parent compound were about 1500 and 400, respectively. On transfer to fresh water, the concentrations of C14 and pyriproxyfen in the body decreased rapidly, with half-lives of about 1 and 0.5 days, respectively. The major metabolic pathways were hydroxylation at the 4'-position of the phenoxyphenyl group and conjugation of the resultant phenol with glucuronic acid or sulfuric acid. Cleavage of the ether linkages were less significant.

The dragonfly, *Orthetrum albistrum speciosum*, midge, *Chironomus yoshimatsui*, and mosquito, *Culex pipens pallens*, larvae were exposed to C-14 pyriproxyfen at a concentration of 10 ppb under a flow-through system at 25°C for 48 hr (dragonfly and mosquito) or 24 hr (midge). The concentrations of pyriproxyfen in the dragonfly and mosquito reached an equilibrium on day 1. The maximum bioaccumulation ratios for C14 and the parent compound were about 120 and 40 in the dragonfly, 500 and 47 in midge, and 280 and 93 in mosquito, respectively. On transfer to fresh water, the concentrations of C14 and pyriproxyfen in the body decreased rapidly, with half-lives of about 0.52 and 0.13 days in the dragonfly, 0.28 and 0.07 days in midge, and 0.32 and 0.13 days in mosquito, respectively. The major metabolic pathways common in these aquatic insects were hydroxylation at the 4'-position of the phenoxyphenyl group and cleavage of the pyridyloxy ether bond. Conjugations of the resultant phenol and the alcohol were not identified. Metabolic pathways in the aquatic organisms are shown in Figure 8. These pathways are also found in rat metabolism.

Under flow-through system bluegill sunfish *Lepomis macrochirus* were exposed to 0.14-0.15 ppm solution. Bioaccumulation ratios were found to be about 647 and 30 in the visceral and edible tissues, respectively. On transfer to fresh water, residue level in the viscera declined by 90% in 96 hr. Fingerling channel catfish *Ictalurus punctatus* were introduced to the tanks and maintained for as long as 30 days under static conditions, where soils treated with pyriproxyfen at the initial concentration of 0.085 ppm had been aged under aerobic conditions for 14 days and then flooded. No residues of pyriproxyfen were apparent in soil (<0.002 ppm) and in fish (<0.12 ppm) (*79*).

In any aquatic organisms, bioaccumulation ratios are found not to be high and half-lives are very short (less than 1 day) due to easy biodegradability.

Bioavailability. Pyriproxyfen applied to dairy wastewater lagoons at 0.1 kg a.i./ha in single and multiple applications resulted in control of *Culex* sp. larvae for periods of 7 to 68 days. Length of the control period appeared to be related to water quality, with greater residual efficacy in more polluted sources. The active ingredient apparently adsorbed onto organic debris where efficacy remained high in the lagoon even after water was pumped from the lagoon and replenished with untreated wastewater. It is presumed that pyriproxyfen adsorbed onto the organic matter in the feeding zone for ingestion by mosquito larvae and retained biological activity (*82*).

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

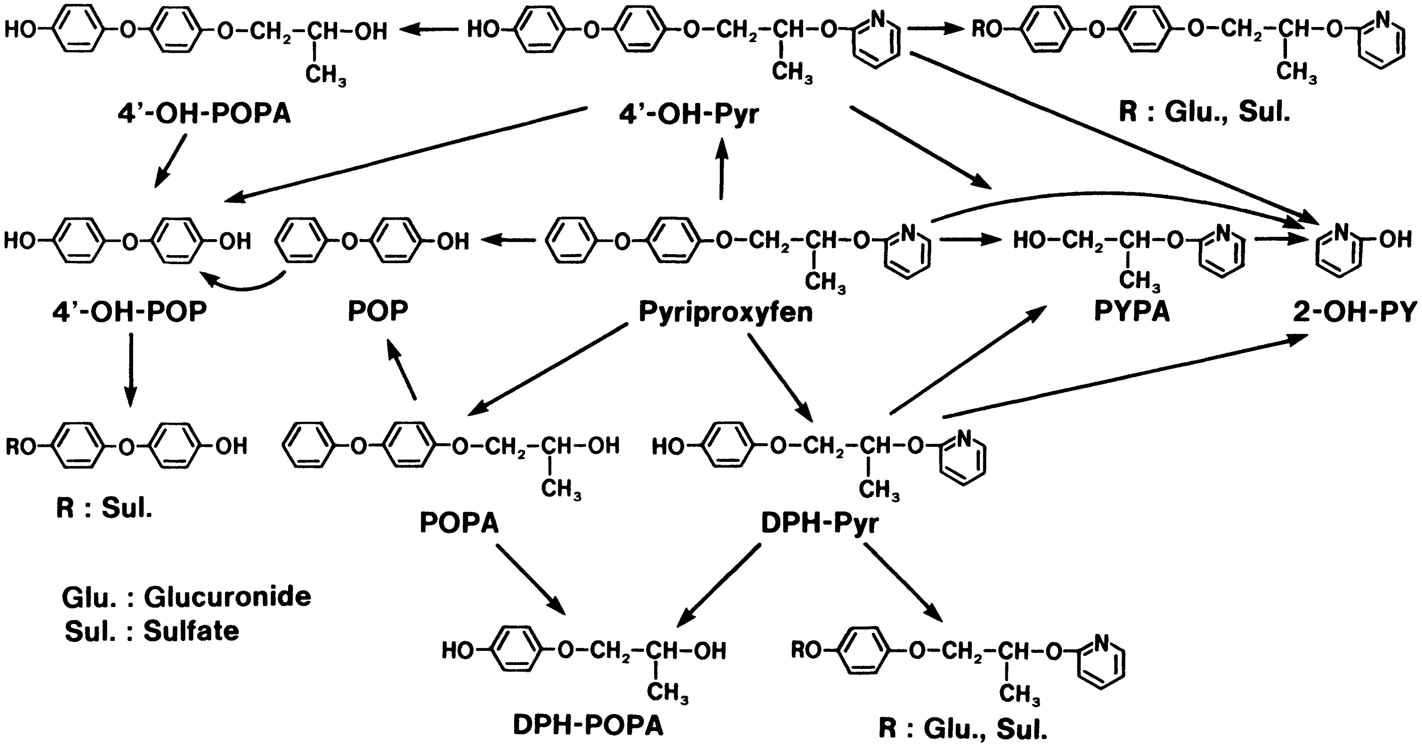

4'-OH-POPA
4'-OH-Pyr
R : Glu., Sul.
4'-OH-POP
POP
Pyriproxyfen
PYPA
2-OH-PY
R : Sul.
POPA
DPH-Pyr
Glu. : Glucuronide
Sul. : Sulfate
DPH-POPA
R : Glu., Sul.

Fig. 8 Proposed metabolic pathways of pyriproxyfen in aquatic organisms including carp, dragonfly, mudge, and mosquito larvae.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

As demonstrated from the field studies, effects on mosquitoes are prolonged for 1-2 months. However, the concentrations of pyriproxyfen in water decreased quite rapidly and no residues were detected within a few days. It is probable that the adosorbed pyriproxyfen on sediments and/or organic matter would be effectively taken up by the target organisms. To verify this speculation, the following studies were carried out, and bioavailability was examined both to target and nontarget aquatic insect larvae.

C-14 pyriproxyfen in 0.5% granule formulation was applied to a water/sediment system (study 1; 20 L water over 1 kg Takarazuka sandy soil and study 2; 10 L over 0.5 kg soil). The system was kept at 25°C under static conditions for 1 day. To eliminate concentration of pyriproxyfen in the water phase, water was passed through at a rate of 20 L or 10 L/day for 8-10 days. Thereafter, insect larvae of dragonfly, *Orthetrum albistrum speciosum*, mosquito, *Culex pipens pallens* and midge, *Chironomus yoshimatsui* were introduced into the system, being kept free or in a cage which was far from the sediment. After a 2-day exposure the insects were analyzed for C14. Table VII clearly shows that uptake of pyriproxyfen from the sediment occurs in mosquitoes and midges, but not dragonfly. This finding demonstrates that pyriproxyfen shows long-term efficacy to target organisms due to bioavailability from sediment and that species-specificity is not only based on the intrinsic toxic effect obtained from laboratory tests but also on habits of the organisms.

Table VII. Uptake of Pyriproxyfen to Aquatic Insects

Study		Concentration (ug/L, ug/Kg)				
		Water	Sediment	Mosquito larva	Midge larva	Dragonfly larva
1	Free	2.0 (0.2)[a]	338 (152)	508 (148)		58.7 (4.2)
	Caged			202 (34.3)		69.0 (4.1)
2	Free	2.1 (0.04)	653 (424)		753 (40.7)	
	Caged				256 (4.4)	

[a] Concentration of 14C and (Pyriproxyfen)
14C-Pyriproxyfen in 0.5% granule was applied to water/sediment system (Study 1; 20 L water over 1 Kg soil and Study 2; 10 L over 0.5 Kg) at 25 C and the system were kept under static condition for 1 day.
After water was flowed at 20 L or 10 L/day for 8-10 days to eliminate the water concentration of pyriproxyfen, insects were kept free or caged in the systems for 2 days under static condition.

Conclusions

From the foregoing discussion, IGRs do show higher species-specificities, being limited to the arthropods, than the conventional insecticides. Among IGRs, CSIs are generally broad spectrum compounds, affecting molting of arthropods. To develop selectivity of CSIs, the mode of action should be carefully investigated between target insects and at least crustaceans. JHAs are generally quite selective, namely being species- and stage-specific. Nontarget insect larvae at the last stage would suffer metamorphogenic effects, whereas in crustaceans the main effects are on the reproductive systems and are found to be reversible. Many environmental studies have demonstrated that environmental effects are transient and therefore acceptable due to easy degradability, and non-lethal and reversible effects on most of the aquatic arthropods in the aquatic ecosystems. These findings favor the environmental safety for controlling mosquitoes and midges by JHAs, without further large scale experimental trials for evaluation of environmental impact. Although commercialized JHAs apparently have a considerable margin of safety to the environment, to be an integral part of the insecticide spectrum, the potential risks have not been completely evaluated due to the lack of sufficient information on mode of action of JHA as well as endocrinology of arthropods. Therefore, further studies are needed to specify mechanisms controlling JHs and expressing physiology by JHs in arthropods at different divisions. Such research will contribute to developing more ideal insect control agents with reduced environmental impacts.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Literature Cited

1. van Daalen, J.J.; Meltzer, J.; Mulder, R.; Wellingna, K. *Naturwissenschaften* **1972**, *59*, 312.
2. Uchida, M.; Asai, T.; Sugimoto, T. *Agric.Biol.Chem.* **1985**, *49*, 1223.
3. Reynolds, S.E. *Pestic.Sci.* **1987**, *20*, 131.
4. Fox, P. *Insect Growth Regulators*; PJB Publications Ltd.: Richmond, 1990; pp 1-102.
5. Hashizume, B. *Japan Pestic. Information* **1988**, *53*, 32.
6. Shibuya, M. *Japan Pestic. Information* **1984**, *44*, 17.
7. Mitsui, T.; Nobusawa, C.; Fukami, J.; Colins, J.; Riddiford, L.M. *J. Pesticide Sci.* **1980**, *5*, 335.
8. Mitsui, T.; Nobusawa, C.; Fukami, J. *J. Pestic. Sci.* **1981**, *6*, 155.
9. Mitsui, T.; Tada, M.; Nobusawa, C.; Yamaguchi, I. *J. Pestic. Sci.* **1985**, *10*, 55.
10. Deul, D.H.; de Jong, B.J.; Kortenbach, J. A. M. *Pestic. Biochem. Physiol.* **1978**, *8*, 98.
11. Post, L.C.; Vincent, W. R. *Naturwissenschaften* **1973**, *60*, 431.
12. Clark, B.S.; Jewess, P. *J. Pestic. Sci.* **1990**, *28*, 377.
13. Degheele, D. In *Pestic. Alternatives*; Casida, J.E., Ed.; Elsevior, 1990, pp 377-388.
14. Ishaaya, I.; Casida, J.E. *Pestic. Biochem. Physiol.* **1974**, *4*, 484.
15. Mayer, R.T.; Chen, A.C.; DeLoach, J.R. *Experientia* **1981**, *37*, 337.
16. Cohen, E.; Casida, J.E. *Pestic. Biochem. Physiol.* **1982**, *17*, 301.
17. Leighton, T.; Marks, E.; Leighton, F. *Science* **1981**, *213*, 905.
18. Marks, E.P.; Leighton, T.; Leighton, F. In *Insecticide Mode of Action;* Coats, J.R., Ed., Academic Press: New Yoyk, 1982, pp 283-313.
19. Williams, C.M. *Nature* **1956**, *178*, 212.
20. Williams, C.M. *Sci. Am.* **1967**, *217*, 13.
21. Henrick, C.A. In *Insecticide Mode of Action*; Coats, J.R., Ed., Academic Press: New York, 1982, pp 315-402.
22. Mulla, M.S.; Darwazeh, H.A.; Kennedy, B.; Dawson, D.M. *J. Am. Mosq. Control Assoc.* **1986**, *2*, 314.
23. Estrada ,J.G.; Mulla, M.S. *J. Am. Mosq. Control Assoc.* **1986**, *2*, 57.

24. Iwanaga, K.; Kanda, T. *Appl. Ent. Zool.* **1988**, *23*, 186.

25. Hatakoshi, M.; Kawada, H.; Nishida, S.; Kisida, H.; Nakayama, I. *J. Sanit. Zool.* **1987**, *38*, 271.

26. Suzuki, H.; Okazawa, T.; Kere, N.; Kawadw, H. *Jpn. J. Sanit. Zool.* **1989**, *40*, 253.

27. Kamimura, K. *Jpn. J. Environ. Entomol. Zool.* **1991**, *3*, 1.

28. Hatakoshi, M.; Shono, Y.; Yamamoto, H.; Hirano, M. *Appl. Ent. Zool.* **1991**, *26*, 412.

29. Yamamoto, H.; Kasamatsu, K. In *Advances in Invertebrate Reproduction*; Hoshi, M.; Yamashita, O.; Ed., Elsevier Science Publisher: Amsterdam, 1990, Vol. 5, pp 393-398.

30. Cooper, R.M.; Oetting, R.D. *J. Entomol. Sci.* **1985**, *20*, 429.

31. McMullen, R.D. In *Advances in Invertebrate Reproduction*; Hoshi, M.; Yamashita, O.; Ed., Elsevier Science Publisher: Amsterdam, 1990, Vol. 5, pp 399-402.

32. Fain, M.J.; Riddiford, L.M. *Biol. Bull.* **1975**, *149*, 506.

33. Riddiford, L.M. *Nature* **1976**, *259*, 115.

34. Hatakoshi, M.; Agui, N.; Nakayama, I. *Appl. Ent. Zool.* **1986**, *21*, 351.

35. Hatakoshi, M.; Nakayama, I.; Riddiford, L.M. *J. Insect Physiol.* **1988**, *34*, 373.

36. Goodman, W.G.; Chang, E.S. In *Comprehensive Insect Physiology Biochemistry and Pharmacology*; Kerkut, G.A.; Gilbert, L.I., Ed., Pergamon Press: Oxford, 1985, Vol. 7; pp 491-510.

37. Palli, S.R.; Osir, E.O.; Eng, W.S.; Boehm, M.F.; Edwards, M.; Kulcsar, P.; Ujvary, I.; Hiruma, K.; Prestwich, G.D.; Riddiford, L.M. Proc. *Natl. Acad. Sci. USA* **1990**, *87*, 796.

38. Riddiford, L.M.; Palli, S.R. In *Molecular Insect Science*; Hagedorn, H.H.; Hildebrand, J.G.; Kidwell, M.G.; Law, J.H., Ed., Plenum Press: New York, 1990, pp 77-81.

39. Palli, S.R.; Riddiford, L.M.; Hiruma, K. *Insect Biochem.* **1991**, *21*,7.

40. Mulla, M.S.; Majori, G.;Arata, A.A. *Residue Rev.* **1979**, *71*, 121.

41. Hammock, B.D.; Quistad, G.B. In *Progress in Pesticide Biochemistry*; Hutson, D.H.; Roberts, T.R., Ed.; John Wiley and Sons, Ltd.: New York, 1981, Vol. 1; pp 2-83.

42. Mian, L.S.; Mulla, M.S. *Residue Rev.* **1982**, *84*, 28.

43. Retnakaran, A.; Granett, J.; Ennis, T. In *Comprehensive Insect Physiology Biochemistry and Pharmacology*; Kerkut, G.A.; Gilbert, L.I., Ed.; Pergamon Press: Oxford, 1985, Vol. 12; pp 530-601.

44. Apperson, C.A.; Schaefer, C.H.; Colwell, A.E. Werner, G.H., Anderson, N.L., Dupras, Jr., E.F.; Longanecker, D.R. *J. Econ. Entomol.* **1978**, *71*, 521.

45. Miura, T.; Takahashi, R.M. *Environ. Entomol.* **1974**, *3*, 631.

46. Miura, T.; Takahashi, R.M. *Proc. Calif. Mosq. Control Assoc.* **1974**, *42*, 177.

47. Nimmo, D.R.; Hamarker, T.L.; Moore, J.C.; Wood, R.A. In *Aquatic Toxicology*; Eaton, J.G.; Parrish, P.R.; Hendricks, A.C., Ed.; American Society for Testing and Materials: Philadelphia, PA, 1980, STP 707, pp 366-376.

48. Christiansen, M.E.; Costlow, Jr. J.D.; Monroe, R.J. *Marine Biol.* **1978**, *50*, 29.

49. Weis, J.S.; Cohen, R.; Kwiatkowsi, J.K. *Aquatic Toxicol.* **1987**, *10*, 279.

50. Ali, A.; Mulla, M.S. *Environ. Entomol.* **1978**, *7*, 21.

51. Colwell, A.E.; Schaefer, C.H. *Proc. Calif. Mosq. Control Assoc.* **1978**, *46*, 111.

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

52. Steelman, C.D.; Farlow, J.E.; Breaud, T.P.; Scilling, P.I. *Mosq. News* **1975**, *35*, 67.
53. Wilkinson, J.D.; Biever, K.D.; Ignoffo, C.M.; Pons, W.J.; Morrison, R.K.; Seay, R.S. *J. Ga. Ent. Soc.* **1978**, *13*, 227.
54. Ables, J.R.; Jones, S.L.; Bee, M.J. *S.W. Ent.* **1977**, *2*, 66.
55. Keever, D.W.; Bradley, J.R.Jr.; Ganyard, M.C. *Environ. Entomol.* **1977**, *6*, 732.
56. Johansen, C.; Mayer, D. *Insect. Acaric. Tests* **1977**, *2*, 83.
57. Schroeder, W.J.; Sutton, R.A.; Beavers, J.B. *J. Econ. Entomol.* **1980**, *73*, 637.
58. Bowers, W.S. In *Advances in Invertebrate Reproduction*; Hoshi, M.; Yamashita, O. Ed.; Elsevier Science Publishers: Amsterdam, 1990, Vol. 5, pp 365-382.
59. Laufer, H.; Albrecht, K.H. In *Advances in Invertebrate Reproduction*; Hoshi, M.; Yamashita, O. Ed.; Elsevier Science Publishers: Amsterdam, 1990, Vol. 5, pp 217-222.
60. Borst, D.W.; Laufer, H.; Landau, M.; Chang, E.S.; Hertz, W.A.; Baker, F.C.; Schooley, D.A. *Insect Biochem.* **1987**, *17*, 1123.
61. Cusson, M.; Yagi, K.J.; Ding, Q.; Duve, H.; Thorpe, A.; McNeil, J.N.; Tobe, S.S. *Insect Biochem.* **1991**, *21*, 1.
62. Miura, T.; Takahashi, R.M. *J. Econ. Entomol.* **1973**, *66*, 917.
63. Christiansen, M.E.; Costlow, Jr., J.D.; Monroe, R.J. *Marine Biol.* **1977**, *39*, 269.
64. Schaefer, C.H.; Miura, T.; Mulligan III, F.S.; Dupras, E.F.Jr. *Proc. Calif. Mosq. Control Assoc.* **1974**, *42*, 140.
65. Norland, R.L.; Mulla, M.S. *Environ. Entomol.* **1975**, *4*, 145.
66. Majori, G.; Bettini, S.; Pierdominici, G. *Mosq. News* **1977**, *37*, 57.
67. Hodek, I.; Ruzicka, Z.; Sehnal, F. *Experientia* **1973**, *29*, 1146.
68. El-Banhawy, E.M. *Entomophaga*, **1977**, *22*, 429.
69. Granett, J. *Canad. Ent.* **1974**, *106*, 695.
70. Granett, J.; Weseloh, R.M. *J. Econ. Entomol.* **1975**, *68*, 577.
71. Vinson, S.B. *J. Econ. Entomol.* **1974**, *67*, 335.
72. McNeil, J. *Science* **1975**, *189*, 640.
73. Cameron, S.A.; Robinson, G.E. *Ann. Entomol. Soc. Am.* **1990**, *83*, 626.
74. Robinson, G.E.; Ratnieks, F.L. *J. Econ. Entomol.* **1987**, *80*, 784.
75. Robinson, G.E. *J. Insect Physiol.* **1985**, *31*, 277.
76. Sasagawa, H.; Sasaki, M.; Okada, I. *Appl. Ent. Zool.* **1989**, *24*, 66.
77. Baker, R.J.; Waller,G.D. *Environ. Entomol.* **1978**, *7*, 569.
78. Hrdy, I.; Skrobal, D. *Apidolgie* **1976**, *7*, 39.
79. Schaefer, C.H.; Miura, T.; Dupras, Jr.,E.F.; Mulligan III, F.S.; Wilder, W.H. *J. Econ. Entomol.* **1988**, *81*, 1648.
80. Schaefer, C.H.; Miura, T. *J. Econ. Entomol.* **1990**, *83*, 1768.
81. Schaefer, C.H.; Dupras, Jr.,E.F.; Mulligan III, F.S. *Ecotoxicol. Environ. Safety* **1991**, *21*, 207.
82. Mulligan III, F.S.; Schaefer, C.H., *J. Am. Mosq. Control Assoc.* **1990**, *6*, 89.

RECEIVED August 1, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 27, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch011

Chapter 12

Recent Progress in Avermectin Research

M. H. Fisher

Merck Sharp and Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ 07065

In 1976 scientists at Merck & Co. Inc. discovered a complex of eight closely related natural products, subsequently named avermectins, in a culture of *Streptomyces avermitilis* MA-4680 (NRRL8165) originating from an isolate by the Kitasato Institute from a soil sample collected at Kawana, Ito City, Shizuoka Prefecture, Japan. They are among the most potent anthelmintic, insecticidal and acaricidal compounds known. Avermectin B_1, under the non-proprietory name abamectin, is widely used as an agricultural miticide and its 22,23-dihydro derivative, ivermectin is used world wide as a broad spectrum endectocide in animals and in man. Recently, an intensive program on avermectin derivatives discovered 4"-deoxy-4"-epimethylamino avermectin B_1 benzoate to be the most potent lepidopteracide known. It is being developed under the non-proprietory name emamectin. Mode of action studies, have revealed a high affinity binding protein in membrane fractions from *Caenorhabditis elegans* and *Drosophila melanogaster*. The use of an affinity probe enabled the isolation of three proteins having molecular weights 8, 47 and 53 kD.

In 1976 scientists at Merck & Co. Inc. discovered a complex of eight closely related natural products, subsequently named avermectins, in a culture of *Streptomyces avermitilis* MA-4680 (NRRL8165) originating from an isolate by the Kitasato Institute from a soil sample collected at Kawana, Ito City, Shizuoka Prefecture, Japan. Their structures are shown in Figure 1 (*1*). They are among the most potent anthelmintic, insecticidal and acaricidal compounds known.

The avermectins (Figure 1) are closely related to another group of pesticidal natural products, the milbemycins, the first examples of which were described by Japanese workers, but later were found to be more abundant in nature than the avermectins *(2-7)*. Milbemycin structures are shown in Figure 2. Interestingly, the milbemycins were first discovered as miticides. Their anthelmintic properties were found only after the anthelmintic activity of the avermectins was demonstrated.

To date, two avermectins, ivermectin and abamectin, have been introduced to date for use in animal and human health. Doramectin (*8*), prepared

0097–6156/93/0524–0169$06.00/0

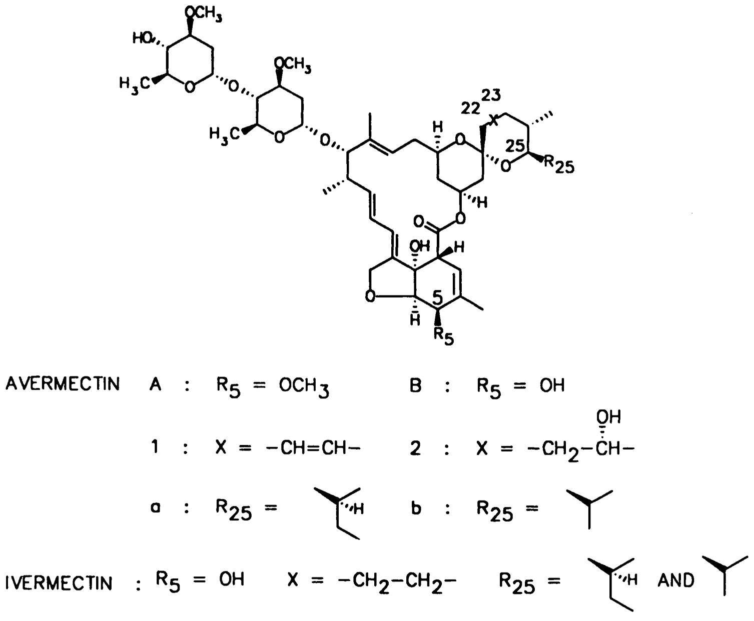
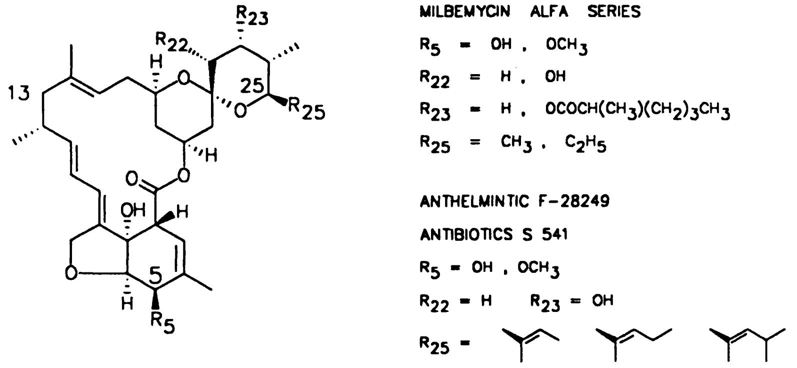

OCH3
HO
H3C
O
OCH3
O
H3C
O
O
22
23
X
25
O
R25
H
O
H
O
O
OH
H
O
5
H
R5
AVERMECTIN A : R5 = OCH3 B : R5 = OH
1 : X = -CH=CH- 2 : X = -CH2-CH(OH)-
a : R25 = b : R25 =
H
IVERMECTIN : R5 = OH X = -CH2-CH2- R25 = AND
H

Figure 1. Avermectin Structures


R23
R22
H
13
O
25
O
R25
H
O
O
OH
H
O
5
H
R5
MILBEMYCIN ALFA SERIES
R5 = OH , OCH3
R22 = H , OH
R23 = H , OCOCH(CH3)(CH2)3CH3
R25 = CH3 , C2H5
ANTHELMINTIC F-28249
ANTIBIOTICS S 541
R5 = OH , OCH3
R22 = H R23 = OH
R25 =

MILBEMYCIN ALFA$_1$ (A$_3$)	R_5 = OH	R_{22} = R_{23} = H	R_{25} = CH_3
MILBEMYCIN ALFA$_3$ (A$_4$)	R_5 = OH	R_{22} = R_{23} = H	R_{25} = CH_2CH_3
MILBEMYCIN D	R_5 = OH	R_{22} = R_{23} = H	R_{25} = $CH(CH_3)_2$
ANTHELMINTIC F-28249-ALPHA	R_5 = OH	R_{22} = R_{23} = H	R_{25} = $C(CH_3)CHCH(CH_3)_2$

Figure 2. Milbemycin Structures

by directed biosynthesis, is expected to be released for use at some future date. Their structures are shown in Figure 3.

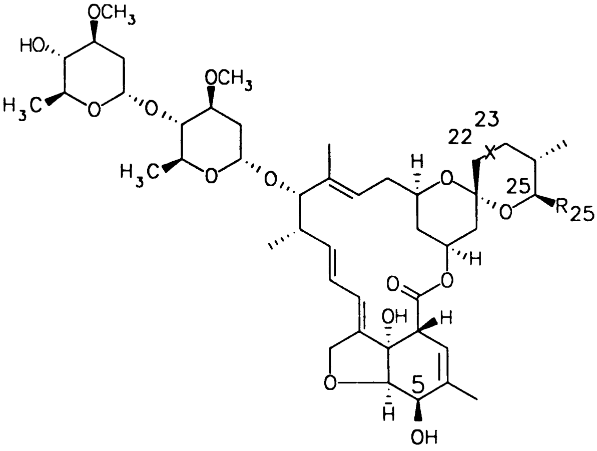
Three milbemycins are used in animal health. Milbemycin D, which is structurally identical to 13-deoxy-22,23-dihydroavermectin B1b aglycone, is used principally in Japan as a canine anthelmintic. Milbemycin oxime is used for the prevention of dog heartworm disease and certain other helminth infections of dogs. Moxidectin is a broad spectrum endectocide, similar to ivermectin in activity. Their structures are shown in Figure 4.

The only avermectin or milbemycin used in agriculture to date is avermectin B1. It has been commercialized for agricultural use under the non-proprietory name abamectin (*Figure 5*).

A summary of the activity of abamectin against some agriculturally important mites and insects is shown in Table I. Inspection of the data shows that it is generally more effective against mites than insects and that its activity against several important lepidopteran species is not commercially viable.

Table I. Activity of Abamectin Against Mites and Insects

Mite Species (Contact effect against adult mites)	**LC_{90} (ppm)**
Phyllocoptruta oleivora (citrus rust mite)	0.02
Tetranychus urticae (two-spotted spider mite)	0.03
Tetranychus turkestani (strawberry mite)	0.08
Panonychus ulmi (European red mite)	0.04
Panonychus citri (citrus red mite)	0.24
Polyphagotarsonemus latus (broad mite)	0.03
Insect Species (Foliar Residue Bioassay)	**LC_{90} (ppm)**
Leptinotarsa decemlineata (Colorado potato beetle)	0.03
Manduca secta (tomato hornworm)	0.02
Epilachna varivestes (Mexican bean beetle)	0.20
Acyrthosiphon pisum (pea aphid)	0.40
Trichoplusia ni (cabbage looper)	1.0
Heliothis zea (corn earworm)	1.5
Spodoptera eridania (southern armyworm)	6.0
Keiferia Lycopersicella (Tomato Pinworm)	0.031

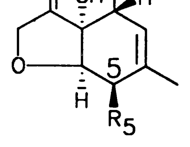

OCH3
HO
H3C
O
OCH3
H3C
O
O
O
22
23
X
H
O
25
O
R25
H
O
O
OH
H
O
5
H
OH

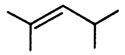
IVERMECTIN : X = $-CH_2CH_2-$ R_{25} = $CH(CH_3)CH_2CH_3$ AND $CH(CH_3)_2$

ABAMECTIN : X = $-CH{=}CH-$ R_{25} = $CH(CH_3)CH_2CH_3$ AND $CH(CH_3)_2$

DORAMECTIN : X = $-CH{=}CH-$ R_{25} = CYCLOHEXYL

Figure 3. Avermectins for Animal and Human Health


23
22
13
H
O
25
O
R25
H
O
O
OH
H
O
5
H
R5

MOXIDECTIN : R_5 = ◀OH R_{23} = $=NOCH_3$ R_{25} = 

MILBEMYCIN OXIME : R_5 = $=NOH$ R_{25} = CH_3 AND C_2H_5

MILBEMYCIN D : R_5 = ◀OH R_{25} = $-CH(CH_3)_2$

Figure 4. Milbemycins for Animal Health

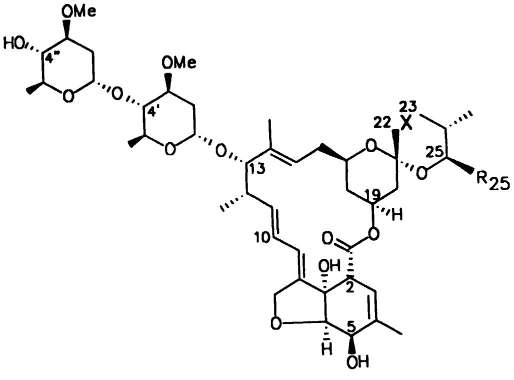
Therefore, an intensive synthetic program was initiated to try to find avermectin derivatives with improved insecticidal activity. The 4"-Epiamino avermectins, prepared by the 4-step synthetic sequence shown in Figure 6 were shown to have the greatest activity. From this series, 4"-Deoxy-4"-epimethylamino avermectin B1 benzoate (MK-244, emamectin)(Figure 7) (*9*) was selected for development. A summary of its activity against insect larvae and adult spider mites and aphids is shown in Table II. The activity of emamectin against lepidoptera is improved up to several hundred fold over abamectin, but its activity against mites and aphids is reduced.

Table II. Foliar Ingestion Activity of 4"-Epi-Methylamino-4"-Deoxyavermectin B_1 Against Insect Larvae and Adult Spider Mites and Aphids

SPECIES (Common Name)	LC_{90}(ppm) at 96 hours
Manduca sexta (L.) (tobacco hornworm)	0.003
Trichoplusia ni (Huebner) (cabbage looper)	0.014
Spodoptera exigua (Huebner) (beet armyworm)	0.005
Spodoptera frugiperda (J.E. Smith) (fall armyworm)	0.01
Leptinotarsa decemlineata (Say) (colorado potato beetle)	0.032
Epilachna varivestis (Mulsant) (Mexican bean beetle)	0.20
Tetranychus urticae (Koch) (two-spotted spider mite)	0.29
Aphis fabae (Scopoli) (bean aphid)	19.9

After several years of avermectin and milbemycin research and synthetic manipulation of most substitutents, little attention had been previously paid to the dioxaspirane system. Figure 8 shows how, starting with avermectin B2a, oxidation to the 23-keto derivative, regioselective enolization followed by epoxidation with MCPBA gave an epoxide which after opening to an α-hydroxyketone was oxidatively cleaved with lead tetraacetate to an intermediate which,when transketalized in acidic methanol, gave a pair of epimeric methoxy ketal-aldehydes (*10*).

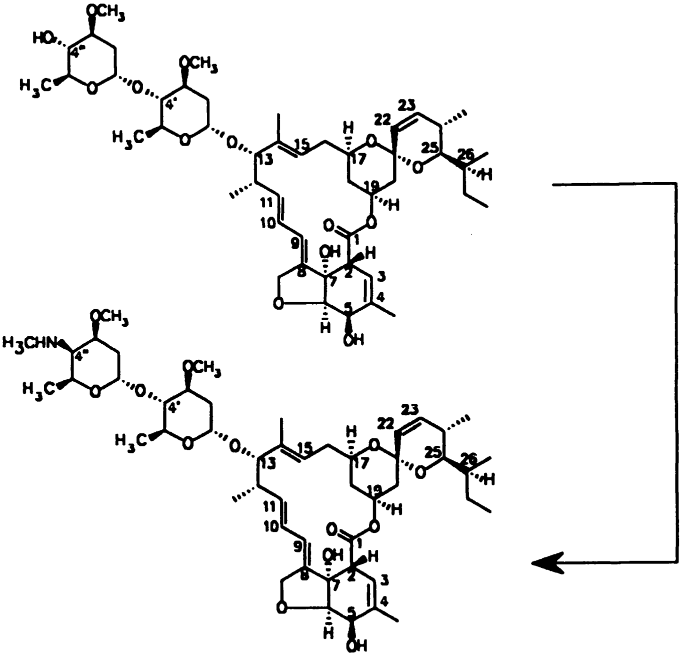
This mixture of methoxyketal-aldehydes provided an extremely useful intermediate for the synthesis of avermectins with novel 24,25 substitutents. Wittig condensation of the mixture with the appropriate phosphonium ylid gave a pair of olefins that were spiroketalized to a single avermectin having the same thermodynamic dioxaspirane configuration as that found in the natural products (*11*) (Figure 9).



ABAMECTIN

R_{25} = $-CH(CH_3)_2$ AND $-CH(CH_3)(C_2H_5)$

Figure 5. Avermectins used in Agriculture



1) t-BUTYLDIMETHYLSILYL CHLORIDE

2) OXALYL CHLORIDE - DMSO

3) H_3CNH_3OAc - $NaBH_4$

4) p-TsOH - H_2O - MeOH

Figure 6. Synthesis of 4"-Epiaminoavermectins

In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.

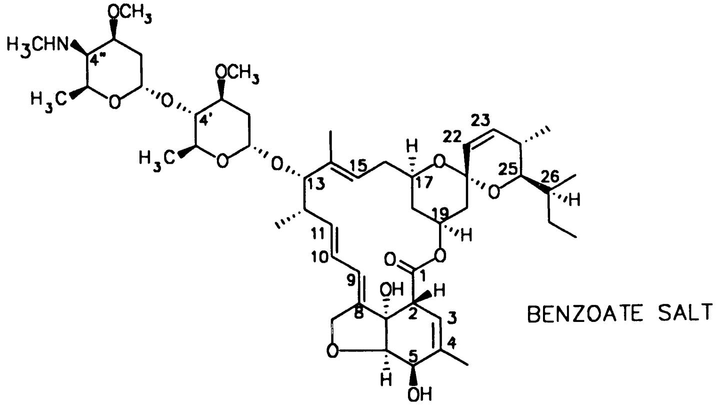

BENZOATE SALT

Figure 7. MK-244 Emamectin

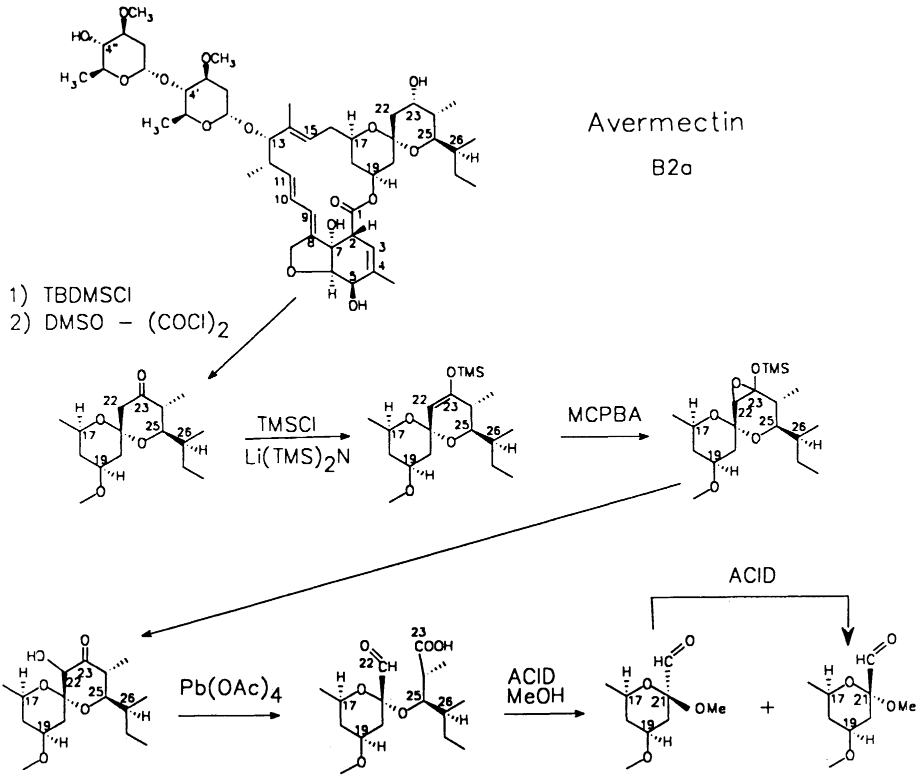

Avermectin
B2a
1) TBDMSCl
2) DMSO – (COCl)$_2$
TMSCl
Li(TMS)$_2$N
MCPBA
Pb(OAc)$_4$
ACID
MeOH
ACID

Figure 8. Cleavage of the Avermectin Spiroketal

Yields of these semi-synthetic avermectins were generally good, as shown in Table III.

Table III. Semi-Synthetic Avermectins

YLID	PRODUCT	YIELD
$Ph_3P=CHCH_2CH_2OSi(Me_2)(t-Bu)$	$R_{24}=H, R_{25}=H$	4%
(R or S)-$Ph_3P=CHCH(Me)CH_2OSiMe_3$	$R_{24}=Me, R_{25}=H$	40%
(S)-$Ph_3P=CHCH_2CHPH(OsiMe_3)$	$R_{24}=H, R_{25}=Ph$	41%
(R,S)-$Ph_3P=CHCH_2CH(OSiMe_3)C_6H_{11}$	$R_{24}=H, R_{25}=$cyclohexyl	39%
(R,S)-$Ph_3P=CHCH_2CH(OSiMe_3)C_5H_9$	$R_{24}=H, R_{25}=$cyclopentyl	40%
(R or S)-$Ph_3P=CHCH_2CH(OSiMe_3)Me$	$R_{24}=H, R_{25}=Me$	45%
$Ph_3P=CHCH(Et)CH(OSiMe_3)(sec-Bu)$	$R_{24}=Et, R_{25}=sec-Bu$	33%
$Ph_3P=CHCH(Me)CH(OSiMe_3)(sec-Bu)$	$R_{24}=Me, R_{25}=sec-Bu$	49%

With a method in hand for the synthesis of novel 24,25-substituted avermectins, we turned our attention to the replacement of the 6,6-spiroketal with a 6,5-system. Starting with the 22,23-hydroxy ketone, oxidation with lead tetraacetate, followed by Horner-Emmons olefination of the adehyde gave an enone which by conjugate reduction of the olefin, followed by reduction to an alcohol, gave an intermediate which could be cyclized to the desired 6,5-spiroketals by acid catalysis under thermodynamic control (*12*). Figure 10 shows the sequence of reactions. Products were obtained in excellent yields as shown in Table IV.

The biological activities of some of the novel 24,25-substituted avermectins and the derivatives containing 6,5-spiroketal systems, as measured by their lethality to brine shrimp, are shown in Tables V and VI.

The mode of action of the avermectins is still incompletely understood. Some pharmacological effects are shown in Table VII. It appears to bind specifically to, and cause opening of, a number of chloride channels at picomolar concentrations (*13*).

A high affinity ivermectin binding site has been found in a membrane fraction from *C. elegans*, the membrane-bound receptor has a kD of 0.14 nm bmax 0.57 p. mol/mg of protein whereas the 1-0-n-octyl-β–D-glucopyranoside (NOG) solubilized receptor has kD 0.18 nm, bmax 0.82 pmol/mg protein. Binding to a similar membrane fraction from rat brain membrane fraction is much less efficient, kD 22 nm which may help to explain the wide margin of safety in mammals (*14*).

In attempt to locate this receptor, an affinity probe, the structure of which is shown in Figure 11 was designed and synthesized (*15*).

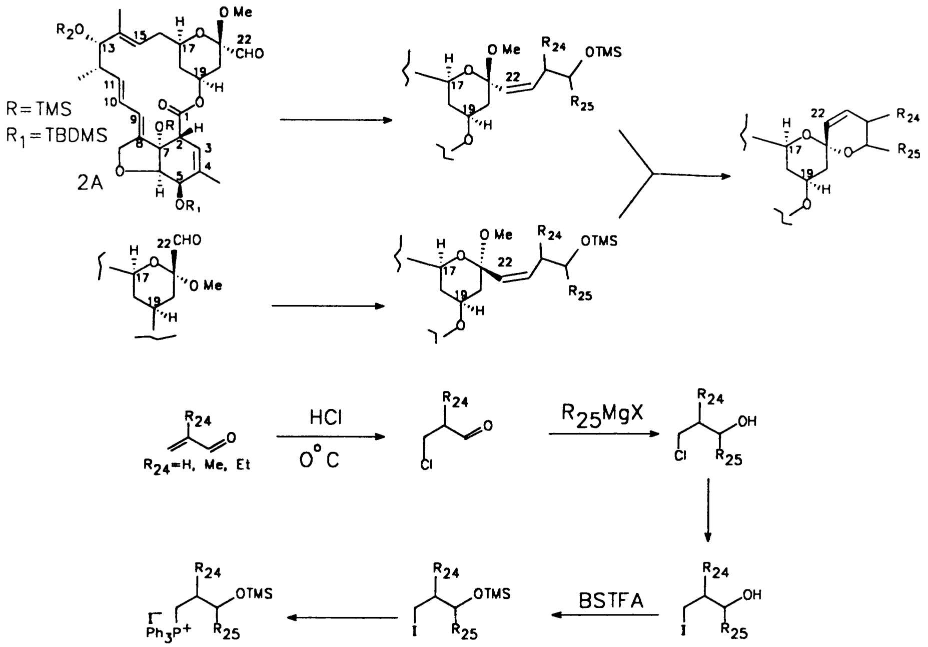
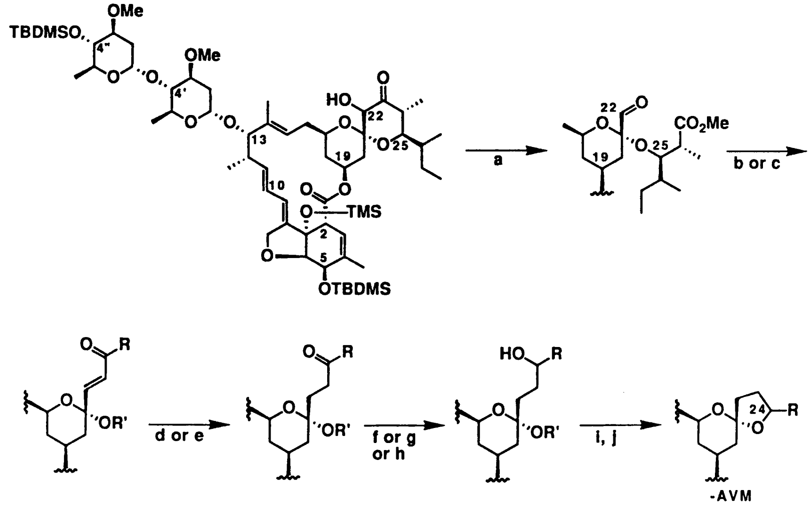

R=TMS
R1=TBDMS
2A
R24=H, Me, Et
HCl
0°C
R25MgX
BSTFA

Figure 9. Wittig Approach to Novel C_{24} - C_{25} Avermectins


TBDMSO
OTBDMS
TMS
a
b or c
d or e
f or g or h
i, j
-AVM

a) $Pb(OAc)_4$, MeOH, pyr; b) $(MeO)_2P(O)CH_2C(O)R$, LiCl, DIEA; c) $Ph_3PCHC(O)R$;
d) 9 eq $Na_2S_2O_4$, 18 eq $NaHCO_3$; 1:1 $PhH:H_2O$, reflux; e) $Pd(PPh_3)_4$, nBu_3SnH;
f) $NaBH_4$; g) RMgBr; h) $BH_3.SMe_2$, oxazaborolidine, 0° C; i) 4:1 PPTS:TsOH; j) HF.pyr

Figure 10. Synthesis of 25-NOR-6, 5-Spiroketal Avermectins

Table IV. 25-OR-24-Substituted Avermectins

R	%Yield	R	%Yield
H	62	CH_2OMe	57
Me	42	CH_2OPh	43
i-pr	68	Ph	38
t-Bu	76	(*p*-F)Ph	30
n-C_6H_{17}	41	(*p*-MeO)Ph	42
c-C_6H_{17}	59	2-Furyl	25
CH_2OH	48	OMe	56

Table V. Activity of Novel 24,25-Substituted Avermectins

24	25	Brine Shrimp LC_{100} - ng/ml
H	H	13,900
CH_3	H	6,930
H	CH_3	1,730
CH_3	CH_3	870
H	sec-Bu	220
CH_3	sec-Bu	220
Et	sec-Bu	870
CH_3	cyclohexyl	1,730
CH_3	phenyl	870

Table VI. Activity of 6,5-Spiroketals

24-R	Brine Shrimp LC_{100} - ng/ml
H	55,500
Methyl	55,500
t-Butyl	870
Phenyl	1,730
p-Fluorophenyl	1,730
p-Methoxyphenyl	6,930
2-Furyl	1,730

Table VII. Pharmacological Effects of Avermectins

Nematodes paralyzed rapidly without causing hypercontraction or flaccid paralysis.
Blockage of signal transmission from ventral interneurons to excitatory motorneurons of *Ascaris*.
Reversible increase of chloride ion permeability of GABA sensitive fibers of the extensor tibiae muscle of the locust *Schistocerca gregaria* at nanamolar concentrations.
Irreversible inhibition of GABA sensitive and insensitive muscle fibers of *Schistocerca gregaria* at micromolar concentrations.
Reversible opening of crayfish stomach chloride channels at subpicomolar concentrations.
Irreversible opening of crayfish stomach chloride channels at 10pmol or higher.
Binds specifically to a number of chloride channel proteins but its binding site is distinct from that of all other effector molecules.

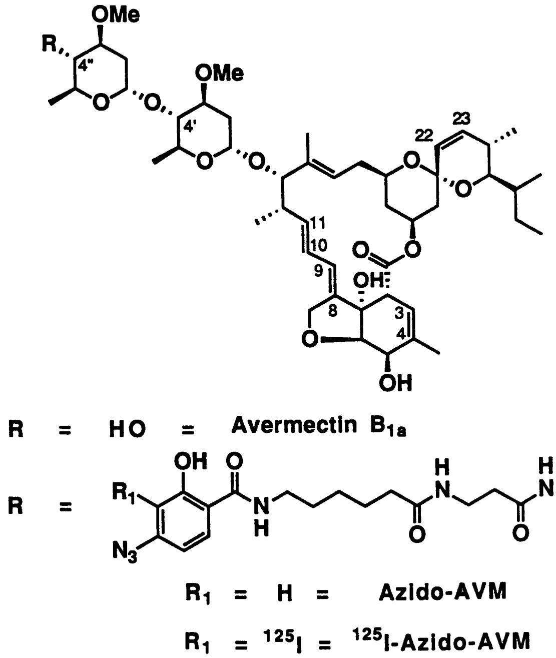


Figure 11. An Affinity Probe for the Avermectin Binding Protein

To demonstrate that azido-AVM retained anthelmintic activity, it was compared to ivermectin in the *C. elegans* motility assay (*16*). The LD_{95}'s were azido-AVM 10ng/ml and ivermectin 3 ng/ml.

Azido-AVM was then shown to bind to triton-solubilized *C. elegans* membrane proteins with IC_{50} 0.3 nM, compared to 0.2 nM for ivermectin. The biologically inactive derivative octahydroavermectin which has been used routinely to uncover non-specific binding, did not inhibit the specific binding of either ivermectin or azido-AVM at concentrations up to 100 nM

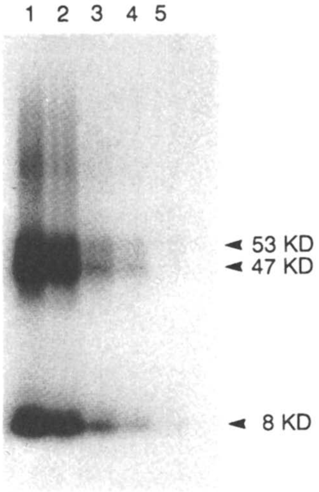
Furthermore, in a competitive binding study, azido-AVM was shown to be a competitive inhibitor of ivermectin with K_i = 0.2 nM.

Similar high-affinity binding was shown for ^{125}I-Azido AVM which bound with kD = 0.48 nM and bmax = 0.38 pmol/mg of protein.

With the assurance that ^{125}I-azido-AVM bound specifically and with high affinity to the ivermectin binding site, labelling studies were undertaken using Triton X-100-solubilized *C. elegans* membrane proteins.

Figure 12 is an autoradiogram of the result obtained when these solubilized proteins were incubated with ^{125}I-azido-AVM in the presence or absence of increasing concentrations of unlabeled ivermectin and then cross-linked with UV light. Three major proteins having molecular weights approximately 8, 47 and 53 kD were revealed. As the concentration of ivermectin during the incubation was increased, the intensity of labelling diminished indicating that the bands were related to the high affinity binding site.

Figure 13 shows a coomassie-stained gel of a similar solubilized-membrane preparation on a 5-20% SDS-polyacrylamide gradient gel. The arrows show where the affinity labelled bands line up with the coomassie-stained bands.


1 2 3 4 5
53 KD
47 KD
8 KD

Ivermectin Concentration

Lane 1	0
Lane 2	2×10^{-10}M
Lane 3	8×10^{-10}M
Lane 4	2×10^{-9}M
Lane 5	2×10^{-8}M

Figure 12. Photoaffinity Labelling of the *C. Elegans* Avermectin Receptor

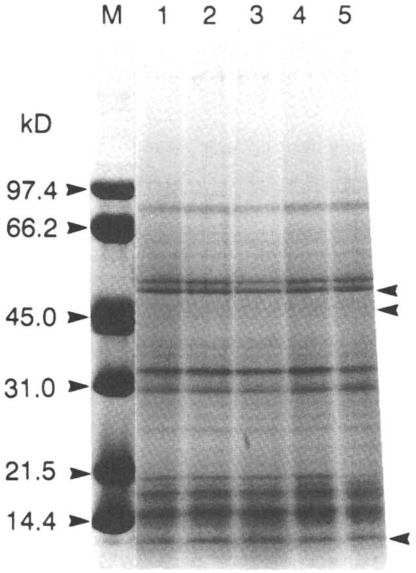

M 1 2 3 4 5
kD
97.4
66.2
45.0
31.0
21.5
14.4

Figure 13. Coomassie Stained Gel of *C. Elegans* Membrane Proteins

In a similar experiment using Drosophila head membranes, a single protein with a molecular weight of approximately 47 kD was labelled (*17*).

Although the mode of action of the avermectins is still not completely understood, isolation and sequencing of the 53, 47 and 8 kD binding proteins from *C. elegans* and the 47 kD protein from *Drosophila melanogaster*, which may turn out to be degradation products of a single protein, takes us one step closer to an understanding.

Literature Cited

1. Albers-Schonberg, G., Arison, B.H., Chabala, J.C. Douglas A. W., Eskola, P., Fisher, M.H., Lusi, A., Mrozik, H., Smith J.L., Tolman, R.L. *J. Am. Chem. Soc.* 1981, 103, 4216.
2. Takiguchi, Y., Mishima, H., Okuda, M., Terao, M., Aoki, A., Fukuda, R. *J. Antibiotics* 1980, 33, 1120.
3. Mishima, H., Ide, J., Muramatsu, S., Ono, M. *J. Antiobiot.* 1983, 36, 980.
4. Wood, I.B., Panksvich, J.A., Carter, G.T., Torrey, M.J., Greenstein, M. European Patent Application EP 170006, 1986.
5. Ward, J.B., Noble, H.M., Porter, N., Flatton, R.A., Noble D. UK Patent Application GB 2166436A, 1986.
6. Carter, G.T., Nietsche, J.A., Borders, D.B. *J. Chem. Soc. Commun.*, 1987, 402.
7. Ramsay, M.J.V., Roberts, S.M., Russell, J.C., Shingler, A.H., Slawin, A.M.Z., Sutherland, D.R., Tiley, E.P., WIlliams, D.J. *Tet. Lett.* 1987, 28, 5353.
8. *USAN and the USP Dictionary of Drug Names*, J.A. Halperin Ed., USP Convention Inc., Rockville, MD, 1991
9. Mrozik, H., Eskola P., Linn B.O., Lusi, A., Tischler, M., Waksmunski, F.S, Wyvratt, M.J., Hilton N.J., Anderson T.E., Babu, J.R., Dybas, R.A., Preiser, F.A., Fisher, M.H., *Experientia,* 1989, 45, 315.
10. Shih, T.L., Mrozik, H., Holmes, M., Fisher, M.H. *Tet. Lett.* 1990, 31, 3525.
11. Shih, T.L., Mrozik, H., Holmes, M., Fisher, M.H. *Tet. Lett.* 1990, 31, 3529.
12. Meinke, P.T., O'Connor, S.P., Mrozik, H. Fisher, M.H. *Tet. Lett.* 1992, 33, 1203.
13. Arena, J.P., Liu, K.K., Paress, P.S., Cully, D.F., in press.
14. Cully, D.F., Paress, P.S. *Mol. Pharmacol.* 1991, 40, 326.
15. Meinke, P.T., Rohrer, S.P., Hayes, E.C., Shaeffer, J.M., Fisher, M.H., Mrozik, H. *J. Med. Chem.*, in press.
16. Schaeffer, J.M., Haines, H.W. *Biochem. Parasitol.* 1989, 38, 2329.
17. Rohrer, S.P., Meinke, P.K., Hayes, E.C., Mrozik, H. Schaeffer, J. *Proc. Nat. Acad. Sci.,* 1992, 89, 4168.

RECEIVED July 29, 1992

Chapter 13

Imidacloprid

A New Nitroguanidine Insecticide

J. W. Mullins

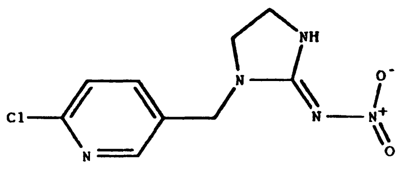
Agriculture Division, Miles Inc., P.O. Box 4913, Kansas City, MO 64120

Imidacloprid (code name: BAY NTN 33893; Chemical Abstract Name: 1-[(6-Chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine) is a highly effective insecticide being developed in the U.S. by Miles Inc. and by Bayer AG worldwide. Imidacloprid is a systemic and contact insecticide exhibiting low mammalian toxicity, with primary activity on sucking insects such as aphids, leafhoppers and planthoppers, thrips and whiteflies, including strains resistant to conventional chemistries. It is also effective against some Coleoptera, Diptera and Lepidoptera. Imidacloprid has a novel mode of action, and no cross resistance from any resistant species has been detected via oral ingestion of imidacloprid in worldwide field and laboratory testing. With excellent systemic and good residual characteristics, imidacloprid is especially appropriate for seed treatment and soil application. Effective early season control with long-lasting protection is achieved in crops such as cereals, corn, cotton, potatoes, rice, sorghum and many vegetables. Pests attacking later in the season can be controlled by foliar applications in the above-mentioned crops, as well as in citrus, deciduous fruits, grapes and other crops. General characteristics of imidacloprid, including biological activity, environmental safety and potential for Insecticide Resistance Management and IPM, are presented and discussed.

The continuing search for new crop protection products to aid in insect pest management led to the discovery of the insecticidal properties of the heterocyclic nitromethylenes by Soloway et. al. *(1)*. Nihon Bayer of Japan synthesized highly active compounds from this chemical group, leading to the development of imidacloprid (proposed common name; code number BAY NTN 33893). Imidacloprid is being cooperatively developed worldwide by Nihon Bayer Agrochem of Japan, Bayer AG of Germany, Miles Inc. Agriculture Division of the United States and Chemagro Limited of Canada.

0097–6156/93/0524–0183$06.00/0

Imidacloprid (designated as a "nitroguanidine") and the closely related nitromethylenes appear to share many similar characteristics including novel mode of action, broad spectrum of activity, low toxicity to vertebrates and favorable environmental fate characteristics, making them desirable candidates as a new generation of insect control agents. The nitromethylenes reported by Soloway et. al. *(1)* have not been developed for general agricultural use due to their extremely rapid degradation under both hydrolytic and photochemical conditions. Relative to the nitromethylenes reported by Soloway, et. al. *(1)*, Imidacloprid is unique in that it exhibits a level of photostability that is appropriate for a variety of uses including outdoor agricultural use.



Imidacloprid

Because of its significant properties, imidacloprid will contribute significantly to efforts to manage insect pests with less environmental impact and increased applicator and consumer safety compared to standard technology. These properties are:

1. Broad spectrum activity/broad crop applicability
2. Favorable characteristics for applicator/consumer/environment
3. A new mode of action for insecticide resistance management
4. A new tool for integrated pest management.

These properties will be discussed in the following sections.

Broad Spectrum of Activity/Broad Crop Applicability

Imidacloprid is a highly effective insecticide exhibiting both systemic and contact activity. The spectrum of activity primarily includes sucking insects (aphids, whiteflies, leaf and planthoppers, thrips, plant bugs, and scales), and also many Coleopteran species (Colorado potato beetle, white grubs, leaf beetles, etc.) and selected species of Diptera and Lepidoptera. Activity has also been demonstrated for ants (Hymenoptera), termites (Isoptera) and cockroaches, grasshoppers and crickets (Orthoptera). No activity has been demonstrated against nematodes or spider mites.

Due to its excellent systemic characteristics, the product is especially appropriate for seed treatment and soil application. Effective early season control with long-lasting protection is achieved in crops such as cereals, corn, rice, potatoes, sugarbeet, and cotton. Pests attacking later in the season can be controlled by foliar applications in these crops, as well as in citrus, deciduous fruits, vegetables, and other crops.

Because imidacloprid is active on many insects that are plant virus vectors, it is effective as a seed or soil treatment in preventing or reducing the spread of many plant viruses such as barley yellow dwarf or curly top of sugarbeets *(2)*. Due to its novel mode of action, imidacloprid is also effective on many insects currently resistant to carbamates, organophosphates or pyrethroids. Excellent crop tolerance and broad spectrum activity allow for the potential use of imidacloprid on a wide range of economically important major and minor crops.

Field use characteristics from a worldwide perspective is discussed by Elbert, et. al. *(2)*. Miles Inc. has conducted field trials in the U.S. to establish dosage rates to further identify the effective pest spectrum and to compare methods and types of application. This testing has provided the information shown in Tables I-IV. These tables represent testing in more advanced stages of development in the U.S. and are not comprehensive lists of test crops. Formulations currently under investigation in the U.S. are listed in Table V. Readers are referred to Dewar et al. *(3)*, Pike et al. *(4)*, Schmeer et al. *(5)*, Almand and Mullins *(6)*, and Oetting *(7)* for discussions on the potential field and greenhouse uses of imidacloprid.

Table I. Activity of Imidacloprid as a Seed Treatment (Food Uses)

Crop	Pest	*Dosage Active Ingredient Metric* g/100 kg seed
Seed Treatment		
Cereals	Aphids (including: Russian wheat aphid English grain aphid Bird oatcherry aphid Greenbug) Hessian Fly	70 to 125
Rice	Rice water weevil	63 to 187
Cotton	Thrips Aphids Leafhoppers Fleahoppers Plant bugs Lygus Whiteflies	63 to 250
* Peanut	Thrips	16 to 63
* Sorghum	Aphids Chinch bug	250 to 1000
* Canola	Flea beetle	500 to 1600

* Require further testing to confirm dosage ranges.

Table II. Activity of Imidacloprid as a Soil Application (Food Uses)

Crop	*Pest*	*Dosage Active Ingredient* *Metric*
		g/meter row
Soil Application In-furrow		
Cotton	Thrips (Pre-squaring) Aphids "	0.01 to 0.03
Potato	Colorado potato beetle Potato leafhopper Green peach aphid	0.01 to 0.03
Peanut	Thrips	0.0075 to 0.015
Sorghum	Aphids (Greenbug, Corn leaf aphid) Chinch bug	0.0075 to 0.03
Soil Application as In-Furrow or Side-Dress		
*Vegetables (Tomato, Pepper, Eggplant, Bean, Onion, Lettuce, Broccoli, Cabbage, Cauliflower, Cucurbits)	Colorado potato beetle Green peach aphid Flea beetle Potato leafhopper Potato aphid Bean leaf beetle Whitefly (bandedwinged & sweetpotato)	0.01 to 0.045
Soil Application as Transplant Drench		
*Vegetables ai/plant (Tomato, Pepper, Eggplant, Bean, Onion, Lettuce, Broccoli, Cabbage, Cauliflower, Cucurbits)	Colorado potato beetle Green peach aphid Flea beetle Potato leafhopper Potato aphid Bean leaf beetle Whitefly (bandedwinged & sweetpotato)	0.005 to 0.04 gm
Tobacco	Aphid Flea beetle	0.01 gm ai/plant
Mechanical or Irrigation Incorporation		
*Pecan	Black pecan aphid Black margined aphid	0.1 to 0.2 gm ai/cm

* Require further testing to confirm dosage ranges.

Table III. Activity of Imidacloprid as a Foliar Treatment (Food Uses)

Crop	*Pest*	*Dosage Active Ingredient* *Metric*
		g/hectare
Foliar Application		
*Cotton	Aphids Whitefly (bandedwinged & sweetpotato)	25 to 150
Potato	Colorado potato beetle Potato leafhopper	25 to 50
Tobacco	Tobacco aphids	50
*Sorghum	Chinch bug Greenbug	50 to 100
Grape	Grape leafhopper	12.5 to 25
*Vegetables (Tomato, Pepper, Eggplant, Bean, Onion, Lettuce, Broccoli, Cabbage, Cauliflower, Cucumber, Squash)	Colorado potato beetle Green peach aphid Flea beetle Potato leafhopper Potato aphid Bean leaf beetle Whitefly (bandedwinged & sweetpotato)	25 to 125
		Concentration
Apple	Rosy apple aphid Green apple aphid Spirea aphid Wooly apple aphid (preventative) Spotted tentiform leaf miner White apple leafhopper	0.003%
*Pecan	Aphids	0.0015% to 0.003%
*Pear	Pear psylla	0.003% to 0.01%
*Citrus	Thrips Scales Root weevils Aphids	0.003% to 0.01%

* Require further testing to confirm dosage rates.

Table IV. Activity of Imidacloprid for Non-Food Uses

Crop	Pest	Dosage Active Ingredient Metric g/hectare
Soil Application		
Turf	White grubs (broadcast)	280 to 560
Ornamentals	Aphids (soil injected) Thrips Leafminers Scales	Rates vary according to pest/plant species.
Foliar Application		
Ornamentals	Aphids Leafhoppers Thrips Scales Psyllids	Rates vary according to pest/plant species.

Table V. Imidacloprid Formulations Tested in the U.S.

Formulation	Target Use
0.62% a.i. GR (Granular)	Turf and Ornamentals
2.5% a.i. GR (Granular)	Agriculture & Ornamentals
240 g a.i./liter FS (Flowable)	Seed Treatment & Liquid Sprays
75% a.i. WS/WP (Wettable Powder)	Seed Treatment & Liquid Sprays
5% a.i. RTU (Ready to Use Liquid)	Trunk or Vine Spray

Favorable Properties for Applicator/Consumer/Environment

Safety to the User and Consumer. Toxicological studies indicate that technical imidacloprid shows low to moderate toxicity to mammals, and all U.S. formulated products currently being registered are proposed as Category III or "Caution" category products (Table VI). One exception is the 2.5 GR formulation which may be rated a category II or "Warning" category. This is based on the slight eye irritation effect of the base clay carrier and not a characteristic of the active ingredient. In acute tests technical imidacloprid shows no eye or dermal irritation, no dermal sensitization and no mutagenic effects.

Chronic toxicity studies have demonstrated that imidacloprid is not carcinogenic, not teratogenic and demonstrates no primary reproductive toxicity. The highly systemic nature of imidacloprid and the favorable toxicological profile are particularly significant because imidacloprid should become a significantly safer alternative to many of the soil applied products currently in use in many crops.

Table VI. Imidacloprid Mammalian Toxicity Results

Formulation	Species	Sex	*Oral LD$_{50}$ mg/kg*	*Dermal LD$_{50}$ mg/kg*	*Inhalation (LC$_{50}$ mg/l) 4 h* *Nominal*	*Analytical*	*EPA Toxicity Category*	*EPA Human Hazard Signal Word*
Technical	Rat	M	424	>5000	>0.5	>0.069	II	WARNING(a)
		F	450-475	>5000	>0.5	>0.069		
					--	>5.3		
					--	>5.3		
2.5% GR, 0.62% GR [1/]								
	Rat	M	>4820		>17.0	>5.1	III	CAUTION(b)
		F	>4820		>17.0	>5.1		(0.62%)
							II	WARNING(c)
	Rabbit	M		>2000				(2.5%)
		F		>2000				
240 FS	Rat	M	>4870		>56.9	>5.3	III	CAUTION(d)
		F	4143		>56.9	>5.3		
	Rabbit	M		>2000				
		F		>2000				
75% WS	Rat	M	2591	>2000	49.2	2.65	III	CAUTION(e)
		F	1858	>2000	45.3	2.75		

[1/] Toxicity data for 0.62% GR is extrapolated from 2.5% GR database.

(a) Proposed Toxicity Category and Human Hazard Signal Word based on inhalation toxicity. The 4 h aerosol inhalation LC$_{50}$ value (analytical >.069mg/l) was the maximum concentration which was technically producible. At this concentration there were no mortalities or symptoms observed. The 4 h inhalation dust LC$_{50}$ value (analytical >5.3 mg/l) is also presented for comparative purposes.

(b) Proposed Toxicity Category and Human Hazard Signal Word based on oral and dermal toxicity and on eye effects.

(c) Proposed Toxicity Category and Human Hazard Signal Word based on eye irritation effects caused by the base clay granule.

(d) Proposed Toxicity Category and Human Hazard Signal Word based on oral and dermal toxicity.

(e) Proposed Toxicity Category and Human Hazard Signal Word based on oral, dermal, and inhalation toxicity.

Use rates of imidacloprid will vary depending on the crop, pest, and type of application; however, most applications will dictate rates that are several times lower than the competitive standards. (See Tables I-IV.) Crop residue studies indicate that both soil and foliar applications result in very low crop residues, generally <1 ppm. Due to the low toxicity of imidacloprid to mammals the proposed reference dose (RfD) is relatively high. Because most treatments with imidacloprid result in low crop residues and the proposed RfD is relatively high, imidacloprid would appear to have the potential to be used on all food crops, including minor crop uses, without exceeding the RfD.

This has been documented by conducting a Chronic Dietary Exposure Analysis *(8)* which estimates the mean chronic exposure to constituents in foods comprising the diets of the average U.S. population and 22 population subgroups. EPA is using this analysis system and designates it as the Dietary Residue Evaluation System or "DRES". This computer model analysis was conducted assuming that all foods consumed contain a tolerance level of imidacloprid residue. A tolerance level for each food crop was estimated from maximum residue levels established in worldwide field residue testing. For crops not tested the tolerance was assumed conservatively to be 1 ppm. Because residue levels seldom reach tolerance levels and normally not every acre of each food crop is treated, actual exposure is generally much less than the calculated value in the model.

The results of the analysis for the U.S. population and some subgroups is presented in Table VII.

Table VII. Imidacloprid Tolerance Assessment Analysis

Population Subgroups	*Percent of ADI*
Nursing infants	20
Non-nursing infants	44
Children (1-6 years)	29
Children (7-12 years)	19
U.S. Population (All Seasons)	13

Given the above parameters, use of imidacloprid on all crops would result in no more than 13% of the total RfD utilized for the general U.S. population. The group commonly considered to be at most risk due to pesticide residues, non-nursing infants, would receive no more than 44% of the total RfD. Given the conservative nature of this analysis and the large margin of safety (100X) associated with the RfD, results indicate that there will be a large measure of safety to the consumer relative to potential residue amounts of imidacloprid on food. Many insecticides are restricted from use on a large number of crops because the total residue consumed would exceed the RfD. Imidacloprid should not be restricted in this sense and could be available for use on any crop, including minor crops, where it is needed.

In assessing the relative safety of imidacloprid to mixer/loader/applicator, estimates of exposure of imidacloprid were derived from surrogate data from previous exposure studies conducted by Miles on other active ingredients, from publications in the literature, and from previous evaluations prepared by EPA's Occupational and Residential Exposure Branch. The use of surrogate exposure data for estimation purposes is based on the premise that mixer/loader and applicator exposures are dependent on the physical parameters (i.e., type of application equipment, application rates, etc.) involved in the application process rather than on the chemical nature of the pesticides themselves. Margins of Safety (MOS) for each type of application relative to inhalation exposure and dermal exposure can be derived by comparing the expected exposure level with the appropriate inhalation or dermal No Effect Levels (NOEL) established in the rat subacute inhalation and rabbit subacute dermal toxicology studies. The low mammalian toxicity of imidacloprid coupled with very low application rates (and corresponding low exposures), result in estimated inhalation MOS that range from 375 to 120,000 and dermal MOS from 7,102 to 333,333 depending on the type of activity (Table VIII). These large margins of safety clearly suggest that imidacloprid can be used safely, without significant risk to people who mix, load or apply it.

Safety to the Environment

Environmental Fate. The dissipation of imidacloprid in the soil environment was investigated in a series of extensive field trials. Field soils, including coarse-textured soils with a high sand content, were treated with the highest proposed labelled rate and residues in soil were measured incrementally over time and at several soil depths. The soil half-life of imidacloprid in each study was < 150 days, but most significantly, no imidacloprid residue was found below the 30.5 cm soil level regardless of soil type. These results indicate that imidacloprid should not leach to groundwater under field conditions, even when used on soils with a high sand content. Efficacy studies with soil applications have demonstrated that, unlike other more soil mobile insecticides, imidacloprid performs best when the product is placed precisely where root uptake can occur. These studies indicate further that imidacloprid does not readily move in the soil (i.e., should not leach).

Crop residue studies have demonstrated low residue from foliar applications. Although these low residue levels are due partly to the low rates applied, residue fate studies conducted on potatoes also indicate a short foliar half-life of approximately one day. In laboratory studies, imidacloprid in water was found to be rapidly degraded by sunlight. This rapid breakdown was confirmed in outdoor aquatic dissipation studies in which the water column half-life was shown to be approximately 1.4 days.

Ecological Effects. Laboratory testing on the standard array of aquatic species indicate unusually low toxicity for an insecticide. Fish LC_{50} values (the concentration required to produce 50% mortality in fish) range from > 105 to 211 ppm. In addition, the LC_{50} for the normally sensitive cladoceran *Daphnia magna* is 85 ppm. Shrimp are considerably more sensitive ($LC_{50} = 0.034$ ppm) and results from studies conducted with other freshwater invertebrates

Table VIII. Imidacloprid Mixer/Loader and Applicator Exposure Estimates and Predicted Margins of Safety for Various Use Patterns

Worker Exposure Activity	*Inhalation Exposure (μg/kg/day)*	*Inhalation Margin of Safety*[1]	*Dermal Exposure (μg/kg/day)*	*Dermal Margin of Safety*[2]
LCO (trigger sprayer)	0.14	17,143	105.7	9,461
MLA (in-furrow planter)	0.60	4,000	3.0	333,333
ML (ground-rig boom)	0.33	72,727	5.94	168,350
A (ground-rig boom)	0.33	72,727	6.49	154,083
MLA (ground-rig boom)	0.05	48,000	9.35	106,951
ML (airblast sprayer)	0.21	11.707	31.8	31,447
A (airblast sprayer)	0.34	7,059	93.7	10,672
MLA (airblast sprayer)	0.27	8,889	62.8	15,924
ML (seed treatment)	2.56	938	140.8	7,102
Coater (seed treatment)	1.60	1,500	44.8	22,321
Bagger (seed treatment)	6.40	375	35.2	28,409
Planter (seed treatment)	0.02	120,000	10.5	95,238

[1] Based on a rat subacute (6 h/day, 5 days/week for 4 weeks) inhalation NOEL of 2.4 mg/kg/day.

[2] Based on a rabbit subacute (6 h/day, 5 days/week for 3 weeks) dermal NOEL of 1000 mg/kg/day.

LCO = Lawn Care Operator, M = Mixer, L = Loader, A = Applicator such as amphipods and chironomids indicate LC_{50}'s in the same range as shrimp. These results led to the conduct of an outdoor microcosm study (10,000-L tanks colonized with aquatic organisms and exposed to varying concentrations of imidacloprid) to evaluate potential effects on invertebrate communities. In this study, temporary reductions in specific aquatic invertebrates were observed at relevant environmental concentrations; however, due to the short half-life in the water column, recovery of the populations was rapid.

The toxicity of imidacloprid to avian species was evaluated in both short and long-term laboratory tests. Minimal effects were observed in bobwhite quail and mallard duck exposed to the compound mixed in the feed. The quail and duck LC_{50} values were 1420 and 5000 ppm, respectively. Long-term reproduction studies (>20 weeks continuous exposure) were also conducted for quail and duck. Reproductive effects were not observed for quail and the No-Observed-Effect-Concentration (NOEC), based on a decrease in adult body weight, was approximately 125 ppm. Based on adverse effects on duck reproduction at 234 ppm, the reproductive NOEC for ducks was also determined to be 125 ppm. Worst case estimates of field exposure via feed were found to be less than 1/10 of the lowest LC_{50} value and less than the NOEC value for chronic and reproductive effects. These values indicate there is minimal hazard to birds from dietary consumption of imidacloprid when used at rates and methods of applications currently being tested (See Tables I-IV).

The other major use pattern for imidacloprid will be seed coatings and soil application of granules. Acute oral toxicity tests conducted with bobwhite quail and house sparrows provided LD_{50} values of 152 and 41 mg/kg, respectively. For the more sensitive songbird species, this is equivalent to the consumption of 419 individual granules. Under field conditions it is highly unlikely that an individual songbird could consume this quantity of granules. However, to confirm the safety of this use pattern, field studies are currently being conducted to evaluate whether field mortality will result during the use of this product.

In summary, extensive laboratory toxicity testing on aquatic and terrestrial species is performed prior to the registration of a new pesticide active ingredient. Based on the results of such testing and on estimates of the concentrations which non-target organisms may be exposed, field tests investigating both fate and effects may also be required. Both laboratory and field tests conducted on aquatic and terrestrial non-target organisms have been performed with imidacloprid and the overall risk assessment indicates that the use of this compound in turf and agricultural crops will result in minimal risk to wildlife.

A New Tool for Resistance Management

Potential for Cross Resistance to Other Insecticides. In numerous worldwide laboratory and field tests no signs of cross-resistance between ingested imidacloprid and conventional insecticides have been detected in any insect species. This has been demonstrated in the field tests with various leafhopper species in Japan, aphid species in Europe, and Colorado potato beetles, greenbugs and other aphid species in the U.S. Control of insects resistant to conventional chemistries is probably due to the unique mode of action of imidacloprid. Like acetylcholine, the nitrometylene analogue, imidacloprid, acts in an agonistic fashion by binding to the nicotinergic acetylcholine receptor in the post-synaptic region of the insect nerve *(9, 10)*. This binding leads to opening of a sodium ion channel. In contrast to acetylcholine, imidacloprid is only slowly degraded in the insect, causing substantial disorder within the nervous system. In most cases this nerve disorder leads to the consequent lethal action. Nicotine, derived from plants, and nereistoxin, originating from a marine worm, have a similar mode of action on insects.

Preliminary studies by Nihon Bayer Agrochem and Bayer AG indicate that organophosphate and carbamate resistant species of leaf and planthoppers were well controlled by the systemic application of imidacloprid and ingestion of the active ingredient *(2)*. However, one organophosphate/carbamate resistant species was less susceptible to topical applications of imidacloprid than were the organophosphate/carbamate sensitive strains of the same species. Several general conclusions can be drawn from these preliminary results. First, an assay method must be developed for each insect species of concern that represents the actual route of exposure (i.e.,best simulates field exposure) to measure the true relative measure of susceptibility among species or strains. Studies with *Myzus persicae* (green peach aphid) indicate that imidacloprid is at least 20X more toxic when administered orally vs. the same amounts administered topically (oral LD_{50} and LD_{95} = 0.5 and 2.0 mg/g, respectively; topical LD_{50} and LD_{95} = 9 and 250, respectively). This basic relationship would appear to extend to most, if not all, insect species. Although conjecture at this time, most of the activity of imidacloprid applied foliarly appears to be due to ingestion of residues primarily, rather than contact exposure. Further research is needed to confirm this premise. Consequently, dosage-mortality assays should be developed to measure toxicity by ingestion and contact activity, rather than contact activity only. Secondly, indications of cross resistance as the result of contact assays only and not feeding assays suggest that reduced penetration may be a possible resistance mechanism. Testing is required to confirm this. Finally, these studies and other studies, including laboratory work with *M. persicae* and field performance studies on a variety of resistant species worldwide indicate no cross resistance has yet been expressed if the insects ingest imidacloprid.

Potential for Resistance Development to Imidacloprid. Beginning in June of 1990, Bayer AG of Germany conducted selection studies on *M. persicae* to determine the potential risk of resistance developing to imidacloprid. The study was initiated using a multi-resistant (OP, carbamate, pyrethroid), greenhouse strain. Beginning in 1990, aphids were exposed to a foliar imidacloprid treatment for 5 days and surviving aphids were placed on untreated cabbage for 9 days. This pattern has been repeated so that the test strain has been exposed to 39 treatments of imidacloprid. Dosage mortality responses were measured periodically throughout the test period. A separate susceptible strain (no imidacloprid treatment) has been maintained for the same time period. Results are summarized in Table IX.

Table IX. Imidacloprid Pressuring Studies on *Myzus persicae* after Approximately Ninety Generations

Rate Applied (% a.i. Concentration)	% Mortality (6 DAT) Susc. Strain	"Pressured" Strain
0.02	100	100
0.004	95	100
0.0008	98	99
0.00016	20	25
0.000032	5	0

Although results of this study and a similar study on planthoppers in Japan do not indicate resistance development to imidacloprid when the insects are allowed to feed on imidacloprid, it remains clear that in principle resistance development against any new insecticide is possible *(11)*. This may especially be true if the target species (aphids, leafhoppers, planthoppers, whiteflies, Colorado potato beetle, etc.) have previously shown a high capability to develop resistance to other chemicals or if use conditions favor repeated applications with only one pesticide. Although further research is needed, early investigations on these aphid and hopper species indicate that insects do not appear to have a high propensity for the development of resistance to imidacloprid, and that imidacloprid will be useful in controlling insects currently resistant to other conventional chemistries. However, to maintain its utility as a resistance management tool, strategies such as insecticide rotations or mixtures should be considered as this novel compound is introduced to the marketplace.

New Tool for IPM

Imidacloprid is effective on a broad range of insects including activity on several beneficial species. But there are several properties of this new chemistry that are favorable for Integrated Pest Management (IPM) efforts. Due to its highly systemic nature and good residual activity, many pests can be controlled with a single soil or seed treatment or trunk spray application. These types of applications would replace single or multiple foliar applications targeted for the same pests. Beneficials that would be killed by foliar applications of imidacloprid or other insecticides would not be exposed to surface residues, and higher levels of beneficials could be maintained while controlling the target pest(s) species. Field experience, however, has demonstrated that in most situations, beneficial insect numbers occur in direct proportion to pest species. When imidacloprid is effective in controlling the pest species that are the primary food source for the beneficials, the number of beneficials could be reduced indirectly due to the lack of a food source.

Although effective on many insect species, imidacloprid is not active on mites. This includes predatory mites which feed on pest mites and some insect pests as well. Neither foliar nor soil applications of imidacloprid affect these beneficial mites. However, occasionally an increase in pest mite numbers will occur after foliar applications of imidacloprid. It is conjectured that this has occurred where the foliar application(s) of imidacloprid has reduced the number of insect predators (leaf-dwelling Coccinellids or thrips) which were the primary biological control factor in maintaining the pest mite population. The effect of foliar applications on many of the parasitic insect species is not fully known; however, field testing results indicate little or no effect on the parasitic beneficials attacking Lepidopteran pests that are not controlled by imidacloprid treatments. Imidacloprid delivered systemically by seed, soil, or trunk paint treatments should have no direct affect on predatory or parasitic species. Bayer AG has demonstrated in laboratory studies that at least one Coccinellid predator species was not affected by feeding on aphids that were exposed to imidacloprid treatments (Leicht, personal communication). Because the amount of imidacloprid necessary to control most pest species by ingestion is so small, a similar safety factor to predator species would be expected for parasitic species present in the host exposed to foliar or systemic applications of imidacloprid. This premise is confirmed by results of field trials in Spanish citrus groves where larval and pupae stages of parasitic Hymenoptera were not harmed by foliar applications of imidacloprid. A U.S. field trial in which apples were treated with imidacloprid resulted in an equal or higher level of parasitism of codling moth larvae (which were not controlled by imidacloprid) compared to the untreated control.

In many field trials excellent plant protection has occurred without a dramatic reduction in insect pest numbers. Laboratory and greenhouse studies conducted to determine how plant protection occurs without a significant reduction in pest numbers have demonstrated that imidacloprid affects many insects species sublethally. These effects are sometimes dosage dependent and include: (1) repellency, (2) reduction or cessation of feeding, (3) reduction or cessation of reproductive activities, (4) overall reduction of movement or activity, and (5) increased susceptibility to biological control.

In many field trials yield protection has occurred without high pest mortality. In principle, maintaining higher pest numbers (while still protecting the plant) should encourage the maintenance of higher beneficial numbers. Laboratory studies have demonstrated with some insect species that sublethal doses of imidacloprid increases their susceptibility to certain microorganisms that are naturally occurring or that could be used commercially in conjunction with imidacloprid treatments. The potential of utilizing the sublethal effects of imidacloprid in conjunction with other control technologies integrated in pest management systems is just beginning to be explored.

The use of imidacloprid will provide the grower with an additional tool for IPM. With a novel mode of action, imidacloprid represents another class of chemistry to include in resistance management. Its versatile application properties will allow the pest management specialist to choose between seed/soil/trunk applications that provide long-lasting control with no surface residues for chronic insect problems, or foliar applications that exhibit shorter residual effectiveness applied on an "as needed" basis only. Although the potential for utilizing the sublethal effects of imidacloprid in conjunction with other control technologies has been indicated, further testing will be needed to understand the full potential.

Summary

The introduction of imidacloprid will represent a major advancement in the ability to manage insect pests in the near future. Due to its unique properties, this new insecticide will enjoy many advantages over current technology in insect control. Its novel mode of action makes it effective on many insect species currently resistant to conventional chemistries. By incorporation into resistance management programs it should become an important new tool to aid in the preservation of susceptibility to insecticides of other classes. Its spectrum of activity and high plant safety characteristics will make it broadly effective for many crops, including minor crops. Its systemic nature and versatile use patterns will allow for new options for Integrated Pest Management. Perhaps most significant is the extremely favorable characteristics relative to the safety of the applicator, consumer, and environment. Although significant advances have been made recently in the development of biologicals and genetic engineering of plants and microbes for insect control, major advances in the development of the more conventional chemistries has been difficult. History indicates that major developments in conventional insecticides, from the carbamates and organophosphates to the pyrethroids, occur only once every two decades. At each step in the development of these chemistries, efforts were directed to provide chemical classes with improved performance, and better human and environmental safety characteristics. As a representative of a new class of insecticides, imidacloprid may provide the next major step forward in this endeavor.

Acknowledgments

The author would like to acknowledge Dr. S. Tsuboi of Nihon Bayer Agrochem for providing data on leaf and planthopper resistance studies from Japan; Drs. C. Erdelen, P. Meisner and W. Leicht for providing data on mode of action and resistance studies conducted at Bayer AG in Germany, and Dr. H. Schmidt of Bayer AG for providing data and general comments on imidacloprid effects on beneficials. The author would also like to thank Dr. R. L. Graney of Miles Inc. for his contributions in the ecological effects discussion, Drs. A. D. Cohick and J. Schneider-Christians of Miles Inc, and A. Klose and A. Elbert of Bayer AG for their review of this manuscript.

Literature Cited

1. Soloway, S. B.; Henry, A. C.; Kollmeyer, W. D.; Padget, W. M.; Powell, J. E.; Roman, S. A.; Tieman, C. H.; Corey, R. A.; Horne, C. A. 1979. Nitromethylene Insecticides. *Advances in Pesticide Science, Fourth International Congress of Pesticide Chemistry*, Zurich, Switzerland, July 24-28, 1978, Part 2, pp 206-217.
2. Elbert, A.; Overbeck, H.; Iwaya, Ki; Tsuboi, S. 1990. Imidacloprid, a novel systemic nitromethylene analogue insecticide for crop protection. *Proceedings of the 1990 Brighton Crop Protection Conference* - Pest and Diseases:21-28.
3. Dewar, A. M.; Read, L.A. 1990. Evaluations of an insecticidal seed treatment, imidacloprid, for controlling aphids on sugar beet. *Proceedings of the 1990 Brighton Crop Protection Conference* - Pest and Diseases:721-726.
4. Pike, K. S.; Reed, G. L.; Graf, G. T.; Allison, D. 1992. Imidacloprid systemic seed treatment effects on germination, plant growth, yield, and compatibility with seed-applied fungicides, and control of Russian wheat aphid in wheat and barley. *Proceedings of the Fifth Russian Wheat Aphid Conference*. Great Plains Agricultural Council Publication No. 142:70-73.
5. Schmeer, H. E.; Bluett, D. J.; Meredith, R.; Heatherington, P.J. 1990. Field evaluation of imidacloprid as an insecticidal seed treatment in sugar beet and cereals with particular reference to virus vector control. *Proceedings of the 1990 Brighton Crop Protection Conference* - Pest and Diseases:29-36.
6. Almand, L. K.; Mullins, J. W. 1991. NTN 33893: a new insecticide for thrips/aphid/whitefly control. *Proceedings of the Beltwide Cotton Production Conference*:80-81.
7. Oetting, R. D. 1990. Imidacloprid for control of whiteflies, *Trialeurodes vaporariorum* and *Bemisia tobaci*, on greenhouse grown poinsettias. *Proceedings of the Brighton Crop Protection Conference* - Pests and Diseases:367-372.
8. TAS: Exposure 1. *Chronic Dietary Exposure Analysis*; Petersen, S. R., Ed.; Technical Assessment Systems, Inc.:Washington, D.C., 1990.
9. Schroeder, M. E.; Flattum, R. F. The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. *Pestic. Biochem. Physiol.* **1984**, *22*, 148-160.
10. Bai, D.; Lummis, S. C. R.; Leicht, V.; Breer, H. and Sattelle, D. B. Actions of imidacloprid and related nitromethylenes on cholinergic receptors of an identified insect motor neurone. *Pestic. Sci.* **1991**, *33*, 197-204.
11. Johnston, A. M.; Lohr, J.; Moes, J.; Soloman, K. R.; Zaborski, E. R. Toxicity of synergized and unsynergized nitromethylene heterocycle insecticide (SD 35651) to susceptible and resistant strains of *Musca domestica* (Diptera:Muscidae). *J. Econ. Entomol.* **1986**, Vol. 79, no. 6:1439-1442.

RECEIVED September 24, 1992

Chapter 14

Application of Semiochemicals in Integrated Pest Management Programs

Louis B. Bjostad, Bruce E. Hibbard, and Whitney S. Cranshaw

**Department of Entomology, Colorado State University,
Fort Collins, CO 80523**

Chemical communication is important in the life cycles of many insect species, principally for the location of mates and food. Structural identification and synthesis has been carried out for many semiochemicals, and a number of techniques have been developed to use these compounds as components of integrated pest management (IPM) programs. Semiochemicals have two main uses in IPM programs: monitoring insect pest populations and preventing agricultural damage by interfering with insect behavior. Pheromones are perhaps the most widely exploited semiochemicals at present, but recent efforts have extended increasingly to the identification and use of compounds involved in interspecific exchange of information as well.

The use of semiochemicals to manipulate insect behavior has proved to be one of the most useful components of integrated pest management (IPM) programs. Semiochemicals are compounds that are emitted or displayed by one organism and that affect the behavior of another organism [*1*]. Perhaps the most important semiochemicals used by insects are those involved in locating mates and food, and at present these are the kinds of semiochemicals most widely exploited for insect control. Semiochemicals with many other kinds of ecological roles have also been characterized in insects, such as compounds that function as warnings, or compounds that mediate the collective activity of many partners in completing large tasks. A number of major reviews of the use of semiochemicals in IPM programs have appeared [*1-9*], as well as a critical review of semiochemical utility in general [*10*]. An extensive list of companies that offer semiochemical lures, traps designed for semiochemical monitoring, and commercial systems for behavioral pest control has been compiled by Inscoe [*11*].

Practical applications of semiochemicals in agriculture fall into two main categories, population monitoring and behavioral pest control. The main use at present is in monitoring insect pests with traps baited with semiochemicals, as a part of making decisions about pesticide application or other control techniques. A second use of semiochemicals is the direct prevention of insect damage by a variety of techniques, including the placement of a large number of semiochemical sources that disrupt the behavior of the insects, mass trapping, attracticides (insecticide lures), and enhancement of parasitoid activity.

0097–6156/93/0524–0199$06.00/0

Although use of semiochemicals in agriculture is centered heavily on pheromones at present, the use of other kinds of semiochemicals in agroecosystems has begun to emerge. Combinations of food-baited and pheromone-baited traps have been evaluated [*12, 13*]. The use of kairomones to control *Varroa* mite, a pest of honeybees, has been proposed [*14*]. Two important groups of semiochemicals that are finding increased use in IPM are anti-feedant compounds [*15-17*] and repellents [*18*].

Formal consideration of semiochemical components of IPM programs is apparent in agricultural systems around the world [*19-24*]. Semiochemical uses in forest IPM have included studies with bark beetles [*25, 26*], ambrosia beetles [*27*], Douglas-fir tussock moth [*28*], pine beauty moth [*29*], and forest defoliators [*30, 31*]. IPM with semiochemicals in fruit agroecosystems has included efforts in apple orchards [*32*] and programs with nitidulid fruit pests [*33, 34*]. Corn IPM has included semiochemical approaches for management of corn rootworms [*35*]. IPM for tsetse fly [*36*] currently includes techniques utilizing semiochemicals.

Monitoring with Semiochemicals

Monitoring insect pest populations with semiochemicals has several powerful advantages over other techniques. Insect sensitivity to semiochemicals is typically very high, and pest populations that have just begun to appear in an area can quickly be detected. The specificity of semiochemicals for particular insect species is also very high, and baited traps can be prepared that will only attract the species of interest. Other monitoring techniques, such as sweep-net sampling or blacklight trapping, usually require more labor or expense to collect the insects, and often require extensive hand-sorting to separate the pest species of interest from many other insects in the sample.

A principal value of monitoring with semiochemicals is the opportunity to reduce the amount of insecticide application but maintain acceptable insect control. Sex pheromone monitoring in the Netherlands and in Canada for the summer tortrix moth (*Adoxophyes orana*), an orchard pest, permitted reduction of insecticide application from 5-7 sprays per season to 3-4 sprays per season [*37, 38*]. In Israel, sex pheromone monitoring for the pink bollworm (*Pectinophora gossypiella*), a cotton pest, has been used in conjunction with a program that has reduced insecticide application from 10-15 sprays per season to no more than 2 sprays per season [*39*].

Types of Monitoring.

Time of First Appearance. Semiochemical trapping can be used qualitatively to determine the presence or absence of pest species. For pests that appear every year in a given crop, semiochemical trapping can be used to determine the time of annual emergence, so that control applications can be timed more accurately. Semiochemical traps have been used to monitor seasonal patterns of appearance for insect pests from a number of insect orders, some recent examples including the boll weevil [*40*], corn earworm [*41*], *Heliothis phloxiphaga* [*41*], sweet potato weevil [*42, 43*], almond seed wasp [*44*], raisin moth [*45*], *Planococcus citri* [*46*], tufted apple bud moth [*47*], rice stem borer [*48*], European corn borer [*49*], bollworm [*50*], and tobacco budworm [*50*], and grape berry moth [*51*].

The codling moth is a major pest in orchards in many parts of the United States, and forecasting systems based on sex pheromone trapping have been developed. The predictive model developed by Riedl [*52*] allows the grower to relate pheromone trap catch to insect emergence and oviposition. The first capture of male codling moths in a pheromone trap is used as a "biofix date", and the accumulation of a specified number of day/degrees after the biofix date is used to estimate first egg hatch (in Michigan, this is an average of 243°C after first trap catch). In a similar effort, predictions based on pheromone trap catches have been compared with the texcim model for predicting *Heliothis* egg timing in cotton [*53*]. Semiochemical monitoring cannot always be used to predict damage in the same season. Gypsy moth (*Lymantria dispar*) larvae begin feeding early in the season, but the adult males do not emerge until several weeks later, and it is therefore not possible to use sex pheromone trapping of adult males for timing insecticide sprays directed against the larvae [*7*].

More recently, the integration of pheromone detection with microbial biocontrol agents rather than chemical pesticides has been developed. Early virus treatment for Douglas-fir tussock moth has been coordinated with pheromone trapping [*54*]. In Kenya, pheromone traps have been used for timing *Bacillus thuringiensis* applications for the African armyworm [*55*].

Exotic Pests. Insect species that have not previously been a pest in a particular geographic area may nevertheless represent such an important threat that monitoring efforts are maintained to detect their presence. The Mediterranean fruit fly is routinely monitored year-round in the U.S. with more than 10,000 traps baited with the chemical attractant trimedlure, as part of an early warning detection system. This monitoring system was an important part of the efforts to eradicate the Mediterranean fruit fly infestation that appeared in the Santa Clara Valley, California, in 1980 [*56*, *57*]. The chemical attractant is a kairomone analog, in that it mimics odors in food hosts for the Mediterranean fruit fly.

The gypsy moth is not a pest in all states, and prior to 1984, gypsy moths had never been found in Colorado. In 1984, members of the National Park Service placed 10 sex pheromone traps for gypsy moths in Rocky Mountain National Park, and a single male was captured throughout the season, the first recorded in Colorado. Extensive pheromone trapping throughout Colorado by the Colorado State Forest Service was initiated, and statewide catches of gypsy moth males were 16 in 1985, 100 in 1986, 384 in 1987, and 194 in 1988. An IPM program for gypsy moth control was conducted in Colorado that included release of sterile gypsy moths, spraying with *Bacillus thuringiensis*, and mass trapping. Pheromone monitoring documented the dramatic drop in Colorado gypsy moth populations, and statewide catches of gypsy moth males were 10 in 1989, 0 in 1990, and 6 in 1991. The rapid detection of the gypsy moth as a new pest in Colorado, and the documentation of its demise in response to a three-part IPM effort, is a dramatic illustration of the sensitivity of semiochemical monitoring [*58*].

Some pest species appear only sporadically as "exotic" pests because they are controlled successfully most of the time by efforts that are actually directed toward other pest species. Prediction of infestations of red scale, which is limited by natural enemies and incidentally by insecticide applications for citrus thrips, has been carried out with pheromone trapping [*59*].

Dispersal and Extent of Distribution. For pests that are principally limited to one portion of a large geographic region, the change in distribution from year to year may be one of the features of greatest interest. The gypsy moth is extensively monitored with semochemicals in states in which it is known as a pest. More than 200,000 traps per year are used throughout the United States for detection and delimitation [*60*]. Regional population trends of gypsy moth in an expanding population have been identified with pheromone trapping [*61*].

The long-range dispersal of the black cutworm has been followed with release-recapture studies [*62*, *63*]. In addition, pheromone trap data have been used in conjunction with National Weather Service atmospheric trajectory data to project the long-range migration of black cutworm adults [*64*]. On a smaller scale, pheromone trap data has been used to evaluate the movement of *Heliothis virescens* in area-wide management programs [*65*, *66*].

Estimate of Density. Semiochemical monitoring is used extensively to determine the presence or absence of a pest population, but the size of the trap catch can also be used to estimate the density of the pest species and determine an economic threshold. Riedl and Croft [*67*] found that the number of codling moths caught in sex pheromone traps could be used to predict the degree of fruit damage that would occur later in the season. Similarly, Sanders [*68*] found that for the spruce budworm, a major forest pest in Canada, pheromone trap catches of adult males could be used to predict the density of larval populations. In Japan, the number of males caught in sex pheromone traps has been used to predict the yield reduction of sweet potatoes by the moth *Spodoptera litura* [*69*].

The use of semiochemical monitoring to determine population density has been questioned in some instances. Alford et al. [*70*] did not find a significant correlation between the number of adult male *Adoxophyes orana* caught in pheromone traps and the amount of fruit damage that later occurred. A possible problem proposed by Audemard [*71*] in using pheromone trap catches of codling moths to predict the population density in an orchard is that semiochemical traps may attract insects from outside the orchard, giving an overestimate of the actual pest density. A solution to this problem was reported by Baker and Roelofs [*72*], who found that the drawing range of trap lures for the Oriental fruit moth *Grapholitha molesta* can easily be controlled by changing the amount of semiochemical in the lure, so that traps only attract males from within the crop area and not from outside it.

The relationship between semiochemical monitoring data and insect density has been considered (in conjunction with a related concept, the effective attraction radius) for a number of bark beetle species [*73*]. In the corn earworm, pheromone catch in cornfields has been related to egg numbers [*74*], and to infestation levels [*75*]. Captures of *Heliothis* species in pheromone traps have been correlated with oviposition in cotton [*76*]. Forecasting larval infestations of pickleworm has also been carried out with sex pheromone trapping [*77*]. The reproductive capacity of *Choristoneura murinana* populations has been estimated not by using only the absolute numbers of males in traps, but by considering the size of the males as well [*78*]. Population densities of stored products insects have also been made with semiochemical monitoring [*79*, *80*]. Pheromone trapping of *Chilo partellus* has been related to male population density and also competition with females [*81*]. Release-recapture data from pheromone traps was used to estimate male density in *Spodoptera litura* [*82*]. Species composition is often of related interest in considering population densities, and sex pheromone traps have been used to determine the relative abundance of *Helicoverpa punctigera* and *Helicoverpa armigera* [*83*], and to determine the relative abundance of *Heliothis* species [*76*].

Insecticide Resistance Monitoring. An innovative development in the use of pheromones is their recent application in detecting the appearance of resistance to pesticides in an insect population in the field. In early trials, pheromone lures were used to attract male moths, and males were then tested for resistance by topical application of pesticide solutions of different concentrations [*84*]. An alternative approach is the use of insecticide-treated vials to test males attracted to pheromone traps [*85*]. In a refinement of this approach, males were trapped in sticky traps baited with pheromone, and topical applications of pesticide solutions were made without removing the males from the glue in the trap [*86*, *87*]. In a further development, pheromone-baited traps have been made with different concentrations of a given pesticide incorporated into the glue in the bottom of a sticky trap, and the numbers of live and dead males have been determined at defined time intervals after capture [*88-90*]. An important element of this technique is the demonstration that adult male susceptibility is highly correlated with larval susceptibility [*91*, *92*].

Quality Control in Biocontrol Programs. Measurement of semiochemical production by insects can be used as an indicator of quality control in the management of insect colonies maintained for use in biocontrol efforts. Pheromone emission in sterile pink bollworms reared for mass release was shown to be competitive with that of wild individuals, as was their mating success [*93*]. Analysis of sex pheromone production for quality control has also been carried out in boll weevil rearing efforts, and selective breeding has even been done to increase the rate of sex pheromone production [*94*]. The role of semiochemicals and foraging behavior with respect to quality of entomophagous insects for biocontrol has been discussed [*95*].

Technical considerations.

Lure Design. Pheromone lures for monitoring are commercially available in several forms, designed to allow semiochemicals to be released at a controlled rate, and often to prevent semiochemical contamination of the user during handling. Semiochemicals are usually non-toxic and do not pose a health risk, but contamination can be a problem if a user handles lures for several different species because cross-contamination of traps can cause reduced trap catches. Current aspects of dispenser designs [*96*] and controlled release formulations [*97*] have been reviewed.

Relative performance of different dispenser types have recently been determined for the pea moth [*98*], the boll weevil [*99*, *100*], the gypsy moth [*101*], Indian meal moth and almond moth [*102*], the Mexican rice borer [*103*], and the Mediterranean fruit fly [*104*, *105*]. A detailed consideration of the release characteristics of a laminated pheromone dispenser for *Helicoverpa zea* has been conducted [*106*, *107*]. Incorporation of pheromone in a starch borate matrix has been used to produce effective lures for western corn rootworm adults [*108*]. Recent developments in lure design include a compressed disk that has been developed for the controlled release of trimedlure C for monitoring of the Mediterranean fruit fly [*109*], and PVC dispensers impregnated with pheromones for tobacco budworm monitoring [*110*, *111*].

Effects of pheromone quantity, age, and environmental exposure of lures are important considerations in planning monitoring applications. Sampling range in relationship to pheromone dosage and lure age has been determined for male sweet potato weevils [*112*]. Evaporation rates from rubber substrates, including a consideration of the effect of temperature, have been examined [*113*, *114*]. Release rates of pheromones from polymeric formulations as a function of temperature and air velocity have also been determined [*115*].

Trap Design. Trap design often has a strong effect on success in designing a monitoring program. McNeil [*8*] recently reviewed the behavioral ecology of chemical communication in moths with respect to trap design. In some cases the absolute efficiences of trap designs have been determined, as for the milk-carton traps frequently used to sample gypsy moths [*116*]. Sticky traps are highly effective in trapping small insects, such as codling moths in apple orchards. Double-cone traps [*117*] are more effective than sticky traps for catching larger moths, such as many species of noctuids, because if their legs become entangled in a sticky trap, they are powerful enough to fly away, leaving the legs behind. Development of trap designs is often based solely on cut-and-try methodology, but the value of behavioral observations in development of new trap designs has proven valuable [*117*, *118*].

In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.

The influence of trap design has been evaluated for many herbivorous pest species, including codling moth and oblique-banded leafroller [*119*], velvetbean caterpillar [*120*], fall armyworm [*120-122*], corn earworm [*123-125*], European corn borer [*126*], black army cutworm [*127*], maize stalk borer [*128*], *Heliothis virescens* [*129, 130*], Mexican rice borer [*131*], *Ephestia kuehniella* [*132*]; hickory shuckworm [*133*], apple leafroller [*134*], Lepidopteran fruit pests [*135*], the noctuid *Mythimna convecta* [*136*], sweet potato weevil [*137*], bark beetles and other forest Coleoptera [*138, 139*], stored products insects [*140-148*], Mediterranean fruit fly [*149*], Mexican fruit fly [*150*] and pine sawfly [*151*]. The influence of trap design is equally important in developing semiochemical monitoring systems for medical and veterinary pests, including sheep blowflies [*152*], disease vectors of horses [*153*], vectors of African swine fever [*154*], mosquito species [*155*], and ticks [*156*].

Innovative trap designs are now commercially available for many specialized monitoring situations. Electronic monitoring systems have been developed in conjunction with semiochemical attraction [*110*]. Photovoltaic-powered suction traps have been developed for weakly flying insects [*157*]. A grain probe trap for stored products insects has been designed as a long cylinder that can easily be inserted into the grain, with small holes just large enough to allow insects (but not grain) to enter the cylinder [*158*].

In some instances, trap designs that were originally developed to rely on physical features such as color and shape for insect monitoring have later been adapted to include semiochemicals as attractive components of the trap. The thigmotactic responses of many stored products pests have been addressed with cardboard traps of appropriate design, and it has been shown that semiochemicals can enhance their effectiveness [*140, 159, 160*]. Semiochemicals have also been shown to augment monitoring of apple maggot flies with sticky spheres [*161*] and yellow panel traps [*162*].

Trap Placement. The context of trap use is often critical to successful monitoring. In field crops such as alfalfa and cotton, sex pheromone sources for adult males of most moth species must be placed near the top of the canopy because the males and females of most species mate at canopy level, and sex pheromone sources scattered on the ground usually have little effect on male behavior. Riedl et al. [*163*] found that the location of sex pheromone traps in apple trees had a significant effect on the numbers of male codling moths caught, and Hirano [*164*] found a significant influence of trap location on sex pheromone attraction of the vegetable pest *Spodoptera litura*. Similar effects in trap placement have been demonstrated for the bollworm [*165*], European corn borer [*166*], corn earworm [*167*], pickleworm [*168*], and *Spodoptera litura* [*169*].

Blend composition. Many semiochemicals consist of a blend of compounds rather than a single compound. The sex pheromones of moths usually consist of blends whose exact proportions are extremely important for optimum attraction. Some semiochemical blends are simpler to identify than others, and blends appropriate for monitoring may undergo revision as more evidence becomes available, as reviewed exhaustively for pheromones [*6*].

The relative efficacies of different commercial pheromone blends have been evaluated for monitoring the blackheaded fireworm, a pest of cranberries [*170*], and for *Heliothis virescens* [*171*]. Similar comparisons have been made with baits for the South American cucurbit fruit fly [*172*]. Field tests of effectiveness have been conducted with new synthetic pheromone blends for the beetle *Prostephanus truncatus*, a stored products pest of maize [*173*]. Undefined chemical blends are of use for some types of monitoring, and factors affecting blend composition have been shown important in these cases as well. The characteristics of hydrolyzed protein baits for flies and other insects are affected by pH [*174*]. A fermentation trap has been developed for selective monitoring of the moth *Mythimna convecta* [*175*].

In some instances, a useful improvement for a pheromone lure is the addition of compounds that are inhibitory to non-target species, and thereby improve the specificity of the lure. Lures for *Grapholitha molesta* that contain a compound inhibitory to *Grapholitha funebrana* have been developed because of their greater specificity [*176*]. Inadvertent capture of non-target species in semiochemical traps has been identified as a significant problem for bark beetles [*177*] and other forest pests [*178*], noctuids in sweet corn [*179*], and corn rootworms [*180*]. The pheromone of the fall armyworm has been implicated in the incidental attraction of bumblebees [*181*].

Behavioral Pest Control

Semiochemicals can be used directly to control insect pest populations by interfering with insect behavior and thereby preventing damage the insects would otherwise do. Semiochemical lures have been developed for dozens of species of insects for population monitoring, but commercial systems that use semiochemicals for direct prevention of insect damage have only been developed for a few insect species. Although behavioral control of insect pest populations has only been developed for a small number of species at present, the systems commercially available for insect control have been found as effective and as economical as conventional control with insecticides. Initial approaches to semiochemical control involved stand-alone applications, in which semiochemicals were evaluated as exclusive agents of pest control. More recently, aproaches have been developed that use semiochemicals as elements of IPM programs. Applications in which semiochemicals, insecticides, biocontrol organisms, and cultural techniques work together are of particular interest, because the special advantages of each can be used to minimize the disadvantages of the others.

Behavioral Disruption. The most common commercial technique for behavioral control of insects is the placement of many individual sources of semiochemical in the host plant to be protected. In a landmark field study led by H. H. Shorey at the University of California at Riverside, it was shown that the pink bollworm, one of the most serious cotton pests in the United States, could be effectively controlled over a large geographic area by placing a large number of synthetic sex pheromone dispensers (hollow plastic fibers) on the cotton foliage [*182*]. Males were unable to locate females to mate because the large number of synthetic pheromone sources effectively competed with the female moths for the attention of the males. Cotton fields treated with sex pheromone alone were protected from larval damage as effectively as fields treated with conventional insecticide applications alone. Moreover, pheromone control was economically competitive with conventional insecticide control. The cost of pheromone control was estimated at $26 per ha per season, which was equivalent to the expense of two insecticide applications. Improvements in control of pink bollworm with mating disruption and with other control techniques in integrated pest management have continued [*183-185*].

The effective use of mating disruption with sex pheromones to control many other pest species of Lepidoptera has now been established, including the Oriental fruit moth [*186*], codling moth [*187*], grape root borer [*188*], gypsy moth [*189*, *190*], lesser peach tree borer [*191*], almond moth [*192*], *Spodoptera litura* [*193*], Douglas-fir tussock moth [*194*], light brown apple moth [*195*], grape berry moth [*196*], *Symmetrischema plaesiosema* [*197*], beet armyworm [*198*, *199*]. The use of pheromone disruption of mating as a component of resistance management efforts has been developed for the light-brown apple moth [*200*], a complementary approach to the use of pheromones in monitoring insecticide resistance in field populations as discussed above. Many different types of dispensers are now commercially available for use as multiple release sources. An extensive list of companies that market dispensers for semiochemical disruption of behavior has been published [*11*]. A polyethylene pheromone dispenser has recently been developed for mating disruption of codling moth [*201*].

Bark beetles coordinate their mass attacks of host tree species by the use of a complex sequence of chemical signals at different stages of the attack, and interference with different steps in the sequence have been exploited for forest protection [*202*]. Use of synthetic semiochemicals to disrupt beetle communication and hinder successful mass attack has been carried out successfully in the mountain pine beetle [*203-206*] and the southern pine beetle [*207*]. The semiochemical blends that are used typically include both pheromones produced by the beetles and kairomones from the tree, but green leaf volatiles alone have been shown to interfere with aggregation in some bark beetles [*208*]. Behavioral disruption with sex pheromones has been carried out with other beetle species, such as the sweet potato weevil [*209-211*].

The physiological mechanisms of mating disruption have been discussed by Renou [*212*]. The role of delayed mating as a factor in success with pheromone disruption has been demonstrated in the stem borer [*213*], and the reproductive effects of mating delay have also been examined in *Pectinophora scutigera* [*214*]. Although the natural blend produced by the female is typically used for mating disruption, the need for this has been questioned [*215*].

As mating disruption has been adopted more widely in agroecosystems, effects extrinsic to control of the target pest species have become of increasing interest. The effects of pheromone mating disruption on parasitoids and other natural enemies of the target pest species has been considered [*216*]. Residue analyses of pheromone components used in grape pest management have been conducted [*217*].

Containment and Concentration. This technique also relies on use of multiple point sources of semiochemicals, but differs from behavioral disruption in that insect attack is not prevented, but is instead redirected to a new location. This approach has been used both for the protection of living trees and harvested trees in lumberyards from attack by the mountain pine beetle [*218*] and the spruce beetle [*219, 220*]. Trap cropping can be considered an instance of containment and concentration with semiochemicals in its simplest form, in that trap crops typically produce semiochemicals that are a central part of their effectiveness [*221*].

Mass Trapping. Although mass trapping appears the most straightforward means of controlling insects with semiochemicals, it has not been widely used for insect control. An important reason for this is that sex pheromones that attract males to females are the semiochemicals that have been most fully characterized, but only males can be trapped with such pheromones, and early theoretical work indicated that significant population reductions might require that 80-95% of the males be trapped [*222*]. Moreover, traps can fill with insects and cease to trap additional insects, necessitating tedious replacement or cleaning, and large numbers of traps may be a considerable expense. Because of these possible problems, initial efforts for semiochemical pest control mainly involved dispersal of multiple semiochemical sources to disrupt the chemical communication of the insects without actually trapping them. Despite the potential difficulties described above, some studies have shown that in practice mass trapping may be a promising approach to insect control.

By far the largest mass trapping program ever conducted was a project initiated by Norway to control the spruce bark beetle *Ips typographus*. The project involved 1 million pheromone traps and cost approximately $23 million. In 1979, 3 billion beetles were trapped, and in 1980, 4.5 billion beetles were trapped. Insecticide application was not possible because of concerns about environmental risk, and a decline in the beetle populations has been observed in conjunction with mass trapping alone [*223*]. This demonstration was not complete, in that sampling was not conducted in non-trapped areas for comparison [*7*]. An overview of the program has recently been presented [*224, 225*]. Recent efforts for pest control by mass trapping have been considered for other insect species, including the western corn rootworm [*226*], *Prays citri* [*227*], spruce bark beetles [*228*], and cockroaches [*229*].

Insecticide Lures ("Attracticides"). The use of attractants has been employed for direct control of insects dating to the time of the Roman Empire. Baits used at the time were chemically uncharacterized, such as blood used in flea traps worn by women in the 15th through 18th centuries [*230*] and bran baits that were commonly employed for grasshopper control in the 1930's [*231*]. Because of the benefits of decreased active ingredient required for control and increased specificity in what insects are controlled, interest in characterized and uncharacterized baits is increasing. Lanier [*232*] reviewed the principles of attraction-annihilation.

One of the more promising prospects for new uses of uncharacterized baits is from the use of powdered roots of buffalo gourd, *Cucurbita foetidissima.* Diabroticite beetles feed strongly on substrates that contain cucurbitacins, bitter triterpenoids found in many species of Cucurbitaceae [*233, 234*]. The western and northern corn rootworm beetles are among the most important insect pests in North America. Sutter et al. [*235*] demonstrated that a toxic bait containing powdered roots of buffalo gourd, 1% carbaryl, and a sticky carrier was effective in controlling adults and subsequent egg-laying in a 16 square mile area in South Dakota. Lance and Sutter [*236, 237*] (and references therein) had previously demonstrated the concept in smaller scale trials. The new formulation is on the market under the registered name SLAM [*238*].

One of the traditional methods for controlling grasshoppers and the red imported fire ant has been with toxic baits. The use of fly pupae as carriers for toxic baits for the red imported fire ant [*239*] helped to solve one of the problems with these baits by making them more specific to the ant species targeted. Other studies with red imported fire ant baits have focused on new toxicants for these baits [*240*] and seasonal changes in bait preference [*241*]. Research with grasshopper baits have demonstrated that they have no significant impact on leafcutting bee populations [*242*], and that both sprays and baits are equally effective in controlling rangeland grasshoppers [*243*]. Formulations have also been developed for formulation of entomopoxviruses for grasshopper control [*244*].

Other recent studies with baits include the incorporation of juvenoid bait formulations for the control of cockroaches [*245*], incorporation of entomophagous nematodes into food baits for control of black cutworms and tawny mole crickets [*246*], and development of baits for soil dwelling insects in sunflower [*247*]. Other studies have focused on combining food-baited traps with pheromone-baited traps [*12, 13, 248*].

The use of characterized attractants in insect control began with methyl eugenol in the control of the oriental fruit fly in Hawaii [*249*]. The approach has been used to successfully control a number of fruit fly species [*250*]. Although some of the fruit flies have been traditionally controlled by uncharacterized baits, work is continuing to develop synthetic lures for these species [*251*].

The term "attracticide" was used to refer to formulations that contained chemically characterized attractants and insecticides by Haynes et al. [*88*]. They demonstrated that males of the pink bollworm moth readily contacted pheromone sources containing different insecticides and suffered significant mortality. Their study was prompted by growers who began adding small amounts of insecticide to the adhesive used to hold multiple pheromone release sources in place in a mating disruption application. The success of the growers in achieving enhanced control with this insecticide lure was later verified in controlled tests by Butler and Las [*252*] and by Beasley and Henneberry [*253*]. An advantage of using insecticide in this way is that non-target organisms, such as many beneficial insect species, are much less at risk fiom the insecticide used in this limiting application. Miller et al. [*254*] demonstrated that increasing the attracticide source to 47,424 flakes per hectare did not cause habituation in the male pink bollworm moth. Attracticide formulations have been developed for other Lepidoptera including the cabbage looper [*255*], the navel orangeworm [*256*], and the Mediterranean flour moth [*257*]. Studies with the pink bollworm and the Mediterranean flour moth involved the use of sex pheromones with insecticides, while the attracticide formulations for the cabbage looper and the navel orangeworm involved the use of host attractants with insecticide. Attracticide formulations have also been developed for Coleopteran species. McKibben et al. [*258*] have developed a design for control of the boll weevil using its aggregation pheromone. Dedek et al. [*259*] used pheromones and insecticides for attracting and killing *Ips typographus* in an integrated approach to forest management.

Increase Parasitoid and Predator Searching. Among the novel approaches for use of semiochemicals in IPM programs is their influence on natural enemies of target pests. Manipulation of the behavior of parasitoids [*260, 261*] and predators [*262*] in order to enhance the impact of biocontrol efforts is currently under investigation. These efforts stemmed from an early demonstration by Lewis et al. [*263*] that *Trichogramma*, an egg parasite of *Helicoverpa zea*, is attracted to the sex pheromone of *H. zea* females. *Trichogramma* females use this semiochemical to select a particular area to search for *H. zea* eggs, but recognize the eggs on the basis of other cues. A potential control strategy involves placing *H. zea* sex pheromone in fields in which *Trichogramma* is released as a biocontrol organism, to induce *Trichogramma* to remain in the field and search for *H. zea* eggs.

Anti-Feedants. The use of chemically defined compounds to prevent feeding by crop and veterinary pests is relatively new in IPM programs [*9, 15-17*]. One of the most useful antifeedants under development at present is azadirachtin, a natural product from the neem tree that is active against a wide variety of insect species [*264-267*]. Among other chemically defined antifeedants, limonoids have been shown to have activity for Colorado potato beetle larvae [*268*] and termites [*269*], rotenoids have been shown to have activity for stored products pests [*270*], and decalins have been shown to have activity for spruce budworms [*271*]. Antifeedants have been used against aphid vectors of plant-virus disease [*272*]. A novel application of plant-produced antifeedants is their use to protect packaging materials from stored products insects [*273*].

Repellents. In IPM programs related to animal and human health, DEET and other synthetic repellents have been used extensively against mosquitoes [*274-286*], against blackflies [*287*], and against ticks [*288*]. Recently, the use of natural products as insect repellents has had renewed interest as well. Natural products with insect repelling activity have been derived from arthropods [*18, 289*] and from essential oils [*290-292*]. Honey has been shown to contain bee repellents, and an analysis of these compounds has been made [*293*]. A horticultural oil has been shown to have repellency against whiteflies [*294*]. Further characterization of these compounds may prove a useful complement to the large number of attractant semiochemicals now used in agriculture.

Summary

Semiochemicals have been in widespread use in agriculture for monitoring insect pests for more than 25 years, and have been in commercial use for more than 15 years for insect control through behavioral disruption. Recent efforts in monitoring have included surveys for insecticide-resistant pests, and tracking the long-range dispersal flights of migratory insects. Recent efforts in semiochemical applications for insect control include the use of "attracticide" formulations that combine semiochemical attractants with reduced amounts of insecticide, and semiochemical manipulation of predator and parasitoid behavior to enhance control of the pests they attack. Antifeedants and repellents are under active investigation at present for use as newer components of IPM systems.

Literature Cited

1. Ridgway, R.L.; Silverstein, R.M.; Inscoe, M.N. *Behavior-modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants.*; Marcel Dekker, Inc.: New York, **1990**.
2. *Control of Insect Behavior by Natural Products*; Wood, D.L.; Silverstein, R.M.; Nakajima, M.; Academic press: New York, **1970**.
3. *Chemical Control of Insect Behavior*; Shorey, H.H.; McKelvey, J.J.; John Wiley & Sons: New York, **1977**.
4. *Management of Insect Pests with Semiochemicals*; Mitchell, E.R.; Plenum: New York, **1981**.
5. *Insect Suppression with Controlled Release Pheromone Systems. Vol. 1-2.*; Kydonieus, A.F.; Beroza, M.; CRC Press: Boca Raton, **1982**.
6. Mayer, M.S.; McLaughlin, J.R. *Handbook of Insect Pheromones and Sex Attractants*; CRC Press: Boca Raton, **1991**.
7. Campion, D.G. In *Techniques in Pheromone Research*; Hummel, H. E. Miller, T. A.; Springer-Verlag: New York, **1984**; pp 405-449.
8. McNeil, J.M. *Ann. Rev. Entomol.* **1991**, *36*, 407-430.
9. *Naturally Occurring Pest Bioregulators*; Hedin, P.A.; Amer. Chem. Soc.: Washington, D.C., **1991**.
10. Pickett, J.A. *Bull. Entomol. Res.* **1991**, *81*, 229-232.
11. Inscoe, M.N.; Ridgway, R.L. In *Behavior-modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants.*; Ridgway, R. L.; Silverstein, R. M. Inscoe, M. N.; Marcel Dekker, Inc.: New York, **1990**; pp 631-715.
12. Barclay, H.J.; Haniotakis, G.E. *Researches on Pop. Ecol.* **1991**, *33*, 269-285.
13. Barclay, H.J. *Researches on Pop. Ecol.* **1991**, *33*, 287-306.
14. Anonymous *Bee World* **1990**, *71*, 40-40.
15. Jermy, T. *J. Chem. Ecol.* **1990**, *16*, 3151-3166.
16. Ley, S.V.; Toogood, P.L. *Chem. in Britain* **1990**, *26*, 31-35.
17. Ley, S.V. *Ciba. Foundation. Symp.* **1990**, *154*, 80-98.
18. Blum, M.S.; Everett, D.M.; Jones, T.H.; Fales, H.M. *ACS Symp. Ser.* **1991**, *449*, 14-26.
19. Vrkoc, J. *Chemica Scripta* **1989**, *29*, 407-410.
20. Gould, F. *Annu. Rev. Entomol.* **1991**, *36*, 305-330.
21. Hedin, P.A. *Crit. Rev. Plant. Sci.* **1990**, *9*, 371-379.
22. Hedin, P.A. *ACS Symp. Ser.* **1991**, *449*, 1-11.
23. Tumlinson, J.H. *J. Chem. Ecol.* **1988**, *14*, 2109-2130.
24. Tumlinson, J.H. *Pure & Appl. Chem.* **1989**, *61*, 559-562.

25. Borden, J.H. *Holarctic Ecol.* **1989,** *12,* 501-510.
26. Baader, E.J. *J. Appl. Entomol.* **1989,** *107,* 32172.
27. Lindgren, B.S. *J. Forestry* **1990,** *88,* 8-11.
28. Mason, R.R.; Wickman, B.E. *Forest Ecol. Manag.* **1991,** *39,* 119-130.
29. Evans, H.F.; Stoakley, J.T.; Leather, S.R.; Watt, A.D. *Forest Ecol. Manag.* **1991,** *39,* 19-28.
30. Ravlin, F.W. *Forest Ecol. Manag.* **1991,** *39,* 3-13.
31. Grant, G.G. *Forest Ecol. Manag.* **1991,** *39,* 153-162.
32. Wildbolz, T. *Entomol. Exper. Appl.* **1988,** *49,* 71-74.
33. Bartelt, R.J.; Dowd, P.F.; Plattner, R.D. *ACS Symp. Ser.* **1991,** *449,* 27-40.
34. Bartelt, R.J.; Dowd, P.F. *Abstr. Papers Amer. Chem. Soc.* **1989,** *198,* 61.
35. Levine, E.; Oloumisadeghi, H. *Annu. Rev. Entomol.* **1991,** *36,* 229-255.
36. Colvin, J.; Gibson, G. *Ann. Rev. Entomol.* **1992,** *37,* 21-40.
37. Minks, A.K.; Dejong, D.J. *J. Econ. Entomol.* **1975,** *68,* 729-732.
38. Dejong, D.J. *EPPO Bull.* **1980,** *10,* 213-221.
39. Shani, A. *Third Israeli Meeting on Pheromone Research, Ben-Gurion University of the Negev,* **1982.**
40. Henneberry, T.J.; Bariola, L.A.; Meng, T. *Southwest. Entomol.* **1988,** *13,* 251-259.
41. Hoffmann, M.P.; Wilson, L.T.; Zalom, F.G. *J. Econ. Entomol.* **1991,** *84,* 902-911.
42. Jansson, R.K.; Heath, R.R.; Coffelt, J.A. *Environ. Entomol.* **1989,** *18,* 691-697.
43. Jansson, R.K.; Mason, L.J.; Heath, R.R. *Sweet Potato Pest Management* **1991,** 97-138.
44. Katsoyannos, B.I.; Kouloussis, N.A.; Bassiliou, A. *Entomol. Exper. Appl.* **1992,** *62,* 9-16.
45. Kehat, M.; Eitam, A.; Blumberg, D.; Dunkelblum, E.; Anshelevich, L.; Gordon, D. *Phytoparasitica* **1992,** *20,* 99-106.
46. Hefetz, A.; Tauber, O. *J. Appl. Entomol.* **1990,** *109,* 502-506.
47. Knight, A.L.; Hull, L.A. *Environ. Entomol.* **1989,** *18,* 939-944.
48. Kondo, A.; Tanaka, F. *Appl. Entomol. Zool.* **1991,** *26,* 167-172.
49. Langenbruch, G.A.; Lorenz, N. *J. Plant Diseases & Protection* **1992,** *99,* 80-92.
50. Leonard, B.R.; Ratchford, K.; Pavloff, A.M.; Micinski, S.; Graves, J.B.; Hammond, A.; Burris, E.; Baldwin, J. *J. Entomol. Sci.* **1989,** *24,* 107-116.
51. Biever, K.D.; Hostetter, D.L. *J. Entomol. Sci.* **1989,** *24,* 472-481.
52. Riedl, H.; Croft, B.A. *Management of the Codling Moth in Michigan, 337,* Michigan State Univ. Agric. Exp. Station. East Lansing, MI., **1978.**
53. Witz, J.A.; Hayes, J.L.; Latheef, M.A.; Lopez, J.D.; Proshold, F.I. *Southwest. Entomol.* **1990,** *15,* 217-229.
54. Otvos, I.S.; Shepherd, R.F. *Forest Ecol. Manag.* **1991,** *39,* 143-151.
55. Broza, M.; Brownbridge, M.; Sneh; B. *Crop. Prot.* **1991,** *10,* 229-233.
56. Leonhardt, B.A.; Cunningham, R.T.; Harte, E.M.; McGovern, T.P. *J. Econ. Entomol.* **1986,** *79,* 1050-1054.
57. Scribner, J. *Hortic. Sci.* **1983,** *18,* 47-52.
58. Leatherman, D.A. *State of Colorado Gypsy Moth Program Report,* Colorado State Forest Service, **1991.**
59. Grout, T.G.; Richards, G.I. *J. Appl. Entomol.* **1991,** *111,* 20-27.
60. Kolodny-Hirsch, D.M.; Schwalbe, C.P. In *Behavior-Modifying Chemicals for Insect Management*; Ridgway, R. L.; Silverstein, R. M. Inscoe, M. N.; Marcel Dekker: New York, **1990**; pp 363-385.

61. Gage, S.H.; Wirth, T.M.; Simmons, G.A. *Environ. Entomol.* **1990,** *19,* 370-377.
62. Showers, W.B.; Smelser, R.B.; Taylor, S.E.; Keaster, A.J.; Lopez, J.D.; Robinson, J.F.; Whitford, F. *Environ. Entomol.* **1989,** *18,* 447-458.
63. Showers, W.B.; Smelser, R.B.; Taylor, S.E.; Lopez, J.D.; Keaster, A.J.; Whitford, F.; Robinson, J.F. *Ecology* **1989,** *70,* 987-992.
64. Smelser, R.B.; Showers, W.B.; Shaw, R.H.; Taylor, S.E. *J. Econ. Entomol.* **1991,** *84,* 879-885.
65. Schneider, J.C. *Environ. Entomol.* **1989,** *18,* 868-874.
66. Schneider, J.C.; Roush, R.T.; Laster, M.L.; Kitten, W.F. *Environ. Entomol.* **1989,** *18,* 438-446.
67. Riedl, H.; Croft, B.A. *Can. Entomol.* **1974,** *106,* 525-537.
68. Sanders, C.J. In *Management of Insect Pests with Semiochemicals*; Mitchell, E. R.; Plenum: New York, **1981**; pp 75-91.
69. Nakasuji, F.; Kiritani, K. *Protection Ecol.* **1978,** *1,* 23-32.
70. Alford, D.V.; Carden, P.W.; Dennis, E.B.; Gould, H.J.; Vernon, J.D.R. *Ann. App. Biol.* **1979,** *91,* 165-178.
71. Audemard, H. *Ann. Zool. Ecol. Anim.* **1979,** *11,* 565-586.
72. Baker, T.C.; Roelofs, W.L. *Environ. Entomol.* **1981,** *10,* 211-218.
73. Byers, J.A.; Anderbrant, O.; Lofqvist, J. *J. Chem. Ecol.* **1989,** *15,* 749-765.
74. Latheef, M.A.; Witz, J.A.; Lopez, J.D. *Can. Entomol.* **1991,** *123,* 271-281.
75. Coop, L.B.; Drapek, R.J.; Croft, B.A.; Fisher, G.C. *J. Econ. Entomol.* **1992,** *85,* 240-245.
76. Leonard, B.R.; Pavloff, A.M.; Church, G.; Burris, E.; Graves, J.B. *J. Econ. Entomol.* **1989,** *82,* 574-579.
77. Elsey, K.D.; Klun, J.A.; Schwarz, M. *J. Econ. Entomol.* **1991,** *84,* 1837-1841.
78. Dumerle, P.; Cornic, J.F. *Acta. Oecol.* **1991,** *12,* 369-383.
79. Hagstrum, D.W.; Flinn, P.W.; Subramanyam, B.; Keever, D.W.; Cuperus, G.W. *J. Kans. Entomol. Soc.* **1990,** *63,* 500-505.
80. Subramanyam, B.; Harein, P.K. *J. Econ. Entomol.* **1990,** *83,* 1102-1109.
81. Unnithan, G.C.; Saxena, K.N. *Appl. Entomol. Zool.* **1991,** *26,* 17-28.
82. Wakamura, S.; Kozai, S.; Kegasawa, K.; Inoue, H. *Appl. Entomol. Zool.* **1992,** *27,* 1-8.
83. Wilson, A.G.L.; Morton, R. *Bull. Entomol. Res.* **1989,** *79,* 265-273.
84. Suckling, J.M.; Penman, D.R.; Chapman, R.B.; Wearing, C.H. *J. Econ. Entomol.* **1985,** *78,* 204-207.
85. Plapp, F.W.; Jackman, J.A.; Campanhola, C.; Frisbie, R.E.; Graves, J.B.; Luttrell, R.G.; Kitten, W.F.; Wall, M. *J. Econ. Entomol.* **1990,** *83,* 335-341.
86. Riedl, H.; Seaman, A.; Henrie, F. *J. Econ. Entomol.* **1985,** *78,* 692-699.
87. Knight, A.L.; Hull, L.A. *J. Econ. Entomol.* **1990,** *83,* 1194-1200.
88. Haynes, K.F.; Miller, T.A.; Staten, R.T.; Li, W.-G.; Baker, T.C. *Experientia* **1986,** *42,* 1293-1295.
89. Haynes, K.F.; Miller, T.A.; Staten, R.T.; Li, W.-G.; Baker, T.C. *Environ. Entomol.* **1987,** *16,* 84-89.
90. Knight, A.L.; Hull, L.A. *J. Econ. Entomol.* **1989,** *82,* 1019-1026.
91. Brewer, M.J.; Trumble, J.T. *J. Econ. Entomol.* **1989,** *82,* 1520-1526.
92. Brewer, M.J.; Trumble, J.T.; Alvarado-Rodriguez, B.; Chaney, W.E. *J. Econ. Entomol.* **1990,** *83,* 2136-2146.
93. Vetter, R.S.; Baker, T.C. *Environ. Entomol.* **1990,** *19,* 21-25.
94. McCoy, J.R.; Wright, J.E. *J. Econ. Entomol.* **1990,** *83,* 610-613.
95. Noldus, L.P.J. *J. Appl. Entomol.* **1989,** *108,* 425-451.

96. Leonhardt, B.A.; Cunningham, R.T.; Dickerson, W.A.; Mastro, V.C.; Ridgway, R.L.; Schwalbe, C.P. In *Behavior-modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants.*; Ridgway, R. L.; Silverstein, R. M. Inscoe, M. N.; Marcel Dekker, Inc.: New York, **1991**; pp 113-130.
97. Weatherston, I. In *Behavior-modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants.*; Ridgway, R. L.; Silverstein, R. M. Inscoe, M. N.; Marcel Dekker, Inc.: New York, **1991**; pp 93-112.
98. Horak, A.; Hrdy, I.; Konecny, K.; Vrkoc, J. *Entomol. Exper. Appl.* **1989**, *53,* 125-131.
99. Leggett, J.E.; McKibben, G.H.; Herbaugh, L. *Southwest. Entomol.* **1989**, *14,* 97-110.
100. Leonhardt, B.A.; Dickerson, W.A.; Ridgway, R.L.; Devilbiss, E.D. *J. Entomol. Sci.* **1990**, *25,* 414-426.
101. Leonhardt, B.A.; Mastro, V.C.; Paszek, E.C.; Schwalbe, C.P.; Devilbiss, E.D. *J. Econ. Entomol.* **1990**, *83,* 1977-1981.
102. Mullen, M.A.; Highland, H.A.; Arthur, F.H. *J. Entomol. Sci.* **1991**, *26,* 64-68.
103. Shaver, T.N.; Brown, H.E.; Hendricks, D.E. *J. Chem. Ecol.* **1990**, *16,* 2393-2399.
104. Leonhardt, B.A.; Cunningham, R.T.; Rice, R.E.; Hendrichs, J.; Harte, E.M. *J. Econ. Entomol.* **1989**, *82,* 860-867.
105. Qureshi, Z.A.; Siddiqui, Q.H.; Hussain, T. *J. Appl. Entomol.* **1992**, *113,* 365-367.
106. Lopez, J.D.; Shaver, T.N. *Southwest. Entomol.* **1990**, *15,* 1-8.
107. Lopez, J.D.; Leonhardt, B.A.; Shaver, T.N. *J. Chem. Ecol.* **1991**, *17,* 2293-2305.
108. Meinke, L.J.; Mayo, Z.B.; Weissling, T.J. *J. Econ. Entomol.* **1989**, *82,* 1830-1835.
109. Heath, R.R.; Chambers, D.L.; Tumlinson, J.H.; Landolt, P.J. *J. Econ. Entomol.* **1990**, *83,* 819-822.
110. Hendricks, D.E.; Leonhardt, B.A.; Shaver, T.N. *Southwest. Entomol.* **1989**, *14,* 17-25.
111. Hendricks, D.E.; Shaver, T.N. *Southwest. Entomol.* **1990**, *15,* 245-251.
112. Mason, L.J.; Jansson, R.K.; Heath, R.R. *J. Chem. Ecol.* **1990**, *16,* 2493-2502.
113. McDonough, L.M.; Brown, D.F.; Aller, W.C. *J. Chem. Ecol.* **1989**, *15,* 779-790.
114. McDonough, L.M. *ACS Symp. Ser.* **1991**, *449,* 106-124.
115. Vanderkraan, C.; Ebbers, A. *J. Chem. Ecol.* **1990**, *16,* 1041-1058.
116. Taylor, R.A.J.; McManus, M.L.; Pitts, C.W. *Bull. Entomol. Res.* **1991**, *81,* 111-118.
117. Kaae, R.S.; Shorey, H.H. *Environ. Entomol.* **1972**, 675-677.
118. Phillips, A.D.G.; Wyatt, T.D. *Entomol. Exper. Appl.* **1992**, *62,* 67-74.
119. Vincent, C.; Mailloux, M.; Hagley, E.A.C.; Reissig, W.H.; Coli, W.M.; Hosmer, T.A. *J. Econ. Entomol.* **1990**, *83,* 434-440.
120. Mitchell, E.R.; Heath, R.R.; Agee, H.R. *J. Chem. Ecol.* **1989**, *15,* 1775-1784.
121. Pair, S.D.; Raulston, J.R.; Douce, G.K.; Sparks, A.N.; Sprenkel, R.K.; Sims, S.R.; Carpenter, J.E. *J. Entomol. Sci.* **1989**, *24,* 34-39.
122. Adams, R.G.; Los, L.M.; Murray, K.D. *J. Econ. Entomol.* **1989**, *82,* 285-290.

123. Gauthier, N.L.; Logan, P.A.; Tewksbury, L.A.; Hollingsworth, C.F.; Weber, D.C.; Adams, R.G. *J. Econ. Entomol.* **1991**, *84,* 1833-1836.
124. Gross, H.R.; Carpenter, J.E. *J. Entomol. Sci.* **1989**, *24,* 482-488.
125. Campbell, C.D.; Walgenbach, J.F.; Kennedy, G.G. *J. Agric. Entomol.* **1992**, *9,* 17-24.
126. Gray, M.E.; Walgenbach, D.D.; Carrick, A.; Troxclair, N.N.; Hein, G.L. *J. Econ. Entomol.* **1991**, *84,* 1196-1202.
127. Gray, T.G.; Shepherd, R.F.; Struble, D.L.; Byers, J.B.; Maher, T.F. *J. Chem. Ecol.* **1991**, *17,* 309-316.
128. Medvecky, B.A.; Zalom, F.G.; Williams, W.A. *Tropical Pest Management* **1992**, *38,* 70-74.
129. Hayes, J.L.; Lockley, T.C. *J. Entomol. Sci.* **1989**, *24,* 355-360.
130. Kehat, M.; Mitchell, E.R.; Heath, R.R. *Fla. Entomol.* **1991**, *74,* 362-365.
131. Shaver, T.N.; Brown, H.E.; Bard, J.W.; Holler, T.C.; Hendricks, D.E. *J. Econ. Entomol.* **1991**, *84,* 1216-1219.
132. Buchi, R. *Anzeiger Fur Schadlingskunde Pflanzenschutz Umweltschutz* **1992**, *65,* 7-11.
133. Hendricks, D.E.; Calcote, V.R. *Southwest. Entomol.* **1991**, *16,* 237-241.
134. Bhardwaj, S.P.; Chander, R. *Tropical Pest Management* **1992**, *38,* 61-64.
135. Knodel, J.J.; Agnello, A.M. *J. Econ. Entomol.* **1990**, *83,* 197-204.
136. McDonald, G.; Farrow, R.A. *J. Australian Entomol. Soc.* **1990**, *29,* 113-121.
137. Jansson, R.K.; Mason, L.J.; Heath, R.R.; Sorensen, K.A.; Hammond, A.M.; Robinson, J.V. *J. Econ. Entomol.* **1992**, *85,* 416-423.
138. Ozaki, K.; Maeto, K.; Choldumrongkul, S. *Appl. Entomol. Zool.* **1991**, *26,* 149-150.
139. Chenier, J.V.R.; Philogene, B.J.R. *Can. Entomol.* **1989**, *121,* 159-167.
140. Trematerra, P.; Girgenti, P. *J. Appl. Entomol.* **1989**, *108,* 32496.
141. Dendy, J.; Dobie, P.; Saidi, J.; Sherman, C. *J. Stored Prod. Res.* **1989**, *25,* 187-191.
142. Barak, A.V.; Burkholder, W.E.; Faustini, D.L. *J. Kans. Entomol. Soc.* **1990**, *63,* 466-485.
143. Cuperus, G.W.; Fargo, W.S.; Flinn, P.W.; Hagstrum, D.W. *J. Kans. Entomol. Soc.* **1990**, *63,* 486-489.
144. Faustini, D.L.; Barak, A.V.; Burkholder, W.E.; Leosmartinez, J. *J. Kans. Entomol. Soc.* **1990**, *63,* 539-547.
145. Mueller, D.; Pierce, L.; Benezet, H.; Krischik, V. *J. Kans. Entomol. Soc.* **1990**, *63,* 548-553.
146. Vick, K.W.; Mankin, R.W.; Cogburn, R.R.; Mullen, M.; Throne, J.E.; Wright, V.F.; Cline, L.D. *J. Kans. Entomol. Soc.* **1990**, *63,* 526-532.
147. Wilkin, D.R. *J. Kans. Entomol. Soc.* **1990**, *63,* 554-558.
148. White, N.D.G.; Arbogast, R.T.; Fields, P.G.; Hillmann, R.C.; Loschiavo, S.R.; Subramanyam, B.; Throne, J.E.; Wright, V.F. *J. Kans. Entomol. Soc.* **1990**, *63,* 506-525.
149. Baker, P.S.; Howse, P.E.; Ondarza, R.N.; Reyes, J. *J. Econ. Entomol.* **1990**, *83,* 2235-2245.
150. Montero, C.A.B.; Salas, J.A.S. *Southwest. Entomol.* **1990**, *15,* 327-331.
151. Anderbrant, O.; Lofqvist, J.; Marling, E.; Jonsson, J. *J. Appl. Entomol.* **1989**, *107,* 365-369.
152. Wall, R.; Green, C.H.; French, N.; Morgan, K.L. *Med. Vet. Entomol.* **1992**, *6,* 67-74.
153. Fletcher, M.G.; Turner, E.C.; Perry, B.D.; Hansen, J.W. *J. Amer. Mosquito Control Assoc.* **1988**, *4,* 431-435.

154. Caiado, J.M.; Boinas, F.S.; Melo, M.A.; Louza, A.C. *Preventive Veterinary Medicine* **1990**, *8*, 55-59.
155. Garcia, R.; Colwell, A.E.; Voigt, W.G.; Woodward, D.L. *J. Med. Entomol.* **1989**, *26*, 327-331.
156. Kinzer, D.R.; Presley, S.M.; Hair, J.A. *J. Med. Entomol.* **1990**, *27*, 750-755.
157. Belding, M.J.; Isard, S.A.; Hewings, A.D.; Irwin, M.E. *J. Econ. Entomol.* **1991**, *84*, 306-310.
158. Burkholder, W.E.; Ma, M. *Annu. Rev. Entomol.* **1985**, *30*, 257-272.
159. Pierce, H.D.; Pierce, A.M.; Borden, J.H.; Oehlschlager, A.C. *J. Econ. Entomol.* **1990**, *83*, 273-276.
160. Javer, A.; Borden, J.H.; Pierce, H.D.; Pierce, A.M. *J. Econ. Entomol.* **1990**, *83*, 268-272.
161. Duan, J.J.; Prokopy, R.J. *J. Appl. Entomol.* **1992**, *113*, 271-279.
162. Geddes, P.S.; Flanders, K.L.; Forsythe, H.Y.; Leblanc, J.P.R. *Environ. Entomol.* **1989**, *18*, 510-512.
163. Riedl, H.; Hoying, S.A.; Barnett, W.A.; Detar, J.E. *Environ. Entomol.* **1979**, *8*, 765-769.
164. Hirano, C. *Jap. J. Appl. Ent. Zool.* **1981**, *25*, 272-275.
165. Witz, J.A.; Lopez, J.D.; Goodenough, J.L. *Southwest. Entomol.* **1992**, *17*, 1-6.
166. Derrick, M.E.; Vanduyn, J.W.; Sorenson, C.E.; Kennedy, G.G. *Environ. Entomol.* **1992**, *21*, 240-246.
167. Drapek, R.J.; Coop, L.B.; Croft, B.A.; Fisher, G.C. *Southwest. Entomol.* **1990**, *15*, 63-69.
168. Valles, S.M.; Capinera, J.L.; Teal, P.E.A. *Environ. Entomol.* **1991**, *20*, 1274-1278.
169. Rao, G.V.R.; Wightman, J.A.; Rao, D.V.R. *Insect Science and Its Application* **1991**, *12*, 443-447.
170. Fitzpatrick, S.M.; Troubridge, J.T. *J. Econ. Entomol.* **1992**, *85*, 947-949.
171. Goodenough, J.L.; Hendricks, D.E.; Lopez, J.D. *Southwest. Entomol.* **1989**, *14*, 71-81.
172. Malavasi, A.; Duarte, A.L.; Cabrini, G.; Engelstein, M. *Florida Entomol.* **1990**, *73*, 510-512.
173. Dendy, J.; Dobie, P.; Saidi, J.A.; Smith, J.; Uronu, B. *J. Stored. Prod. Res.* **1991**, *27*, 69-74.
174. Flath, R.A.; Cunningham, R.T.; Matsumoto, K.E.; Mon, T.R.; Binder, R.G. *J. Agric. Food Chem.* **1989**, *37*, 814-819.
175. McDonald, G. *J. Australian Entomol. Soc.* **1990**, *29*, 107-108.
176. Toth, M.; Sziraki, G.; Szocs, G.; Saringer, E. *Agr. Ecosyst. Environ.* **1991**, *35*, 65-72.
177. Trematerra, P.; Daolio, E. *J. Appl. Entomol.* **1990**, *110*, 275-280.
178. Zumr, V.; Stary, P. *J. Appl. Entomol.* **1991**, *112*, 525-530.
179. Weber, D.C.; Ferro, D.N. *J. Econ. Entomol.* **1991**, *84*, 1364-1369.
180. Weissling, T.J.; Meinke, L.J.; Lytle, K.A. *J. Econ. Entomol.* **1991**, *84*, 1235-1241.
181. Gross, H.R.; Carpenter, J.E. *Environ. Entomol.* **1991**, *20*, 377-381.
182. Gaston, L.K.; Kaae, R.S.; Shorey, H.H.; Sellers, D. *Science* **1977**, *196*, 904-905.
183. Critchley, B.R.; Chamberlain, D.J.; Campion, D.G.; Attique, M.R.; Ali, M.; Ghaffar, A. *Bull. Entomol. Res.* **1991**, *81*, 371-378.
184. Flint, H.M.; Yamamoto, A.; Parks, N.J.; Nyomura, K. *Environ. Entomol.* **1990**, *19*, 1845-1851.

185. Sattaur, O. *New Scientist* **1989,** *121,* 35-35.
186. Audemard, H.; Leblon, C.; Marboutie, G.; Neumann, U. *J. Appl. Entomol.* **1989,** *108,* 191-207.
187. Howell, J.F.; Knight, A.L.; Unruh, T.R.; Brown, D.F.; Krysan, J.L.; Sell, C.R.; Kirsch, P.A. *J. Econ. Entomol.* **1992,** *85,* 918-925.
188. Johnson, D.T.; Lewis, B.A.; Snow, J.W. *Environ. Entomol.* **1991,** *20,* 930-934.
189. Webb, R.E.; Leonhardt, B.A.; Plimmer, J.R.; Tatman, K.M.; Boyd, V.K.; Cohen, D.L.; Schwalbe, C.P.; Douglass, L.W. *J. Econ. Entomol.* **1990,** *83,* 910-916.
190. Kolodnyhirsch, D.M.; Webb, R.E.; Olsen, R.; Venables, L. *J. Econ. Entomol.* **1990,** *83,* 1972-1976.
191. Pfeiffer, D.G.; Killian, J.C.; Rajotte, E.G.; Hull, L.A.; Snow, J.W. *J. Econ. Entomol.* **1991,** *84,* 218-223.
192. Prevett, P.F.; Benton, F.P.; Hodges, R.J.; Hall, D.R.; Serodio, R.D. *J. Stored Prod. Res.* **1989,** *25,* 147-154.
193. Rao, S.V.; Ahmed, K.; Azam, K.M. *Current Science* **1989,** *58,* 933-935.
194. Sower, L.L.; Wenz, J.M.; Dahlsten, D.L.; Daterman, G.E. *J. Econ. Entomol.* **1990,** *83,* 1486-1491.
195. Suckling, D.M.; Rogers, D.J.; Shaw, P.W. *Australian J. Zool.* **1990,** *38,* 363-373.
196. Trimble, R.M.; Pree, D.J.; Vickers, P.M.; Ker, K.W. *Can. Entomol.* **1991,** *123,* 451-460.
197. Vanderkraan, C.; Vandeventer, P. *J. Appl. Entomol.* **1990,** *109,* 213-215.
198. Wakamura, S.; Yamashita, I.; Takai, M.; Kozai, S.; Inoue, H.; Kawamura, M.; Kawahara, S. *Appl. Entomol. Zool.* **1989,** *24,* 387-397.
199. Wakamura, S.; Takai, M.; Yamamoto, A.; Inoue, H.; Kawamura, M. *Appl. Entomol. Zool.* **1990,** *25,* 320-323.
200. Suckling, D.M.; Shaw, P.W.; Khoo, J.G.I.; Cruickshank, V. *New Zealand J. Crop & Hortic. Sci.* **1990,** *18,* 89-98.
201. Brown, D.F.; Knight, A.L.; Howell, J.F.; Sell, C.R.; Krysan, J.L.; Weiss, M. *J. Econ. Entomol.* **1992,** *85,* 910-917.
202. Vite, J.P.; Baader, E. *J. Chem. Ecol.* **1990,** *16,* 3031-3041.
203. Lindgren, B.S.; Chong, L.J.; Borden, J.H.; Higgins, C.J.; Cushon, G.H. *Can. J. Forest Res.* **1989,** *19,* 65-68.
204. Amman, G.D.; Schmitz, R.F.; Mcgregor, M.D.; Thier, R.W. *Can. J. Forest Res.* **1989,** *19,* 60-64.
205. Safranyik, L.; Shore, T.L.; Linton, D.A.; Lindgren, B.S. *J. Appl. Entomol.* **1992,** *113,* 391-397.
206. Shea, P.J.; Mcgregor, M.D.; Daterman, G.E. *Can. J. Forest Res.* **1992,** *22,* 436-441.
207. Payne, T.L.; Billings, R.F. *J. Econ. Entomol.* **1989,** *82,* 1702-1708.
208. Dickens, J.C.; Billings, R.F.; Payne, T.L. *Experientia* **1992,** *48,* 523-524.
209. Mason, L.J.; Jansson, R.K. *Fla. Entomol.* **1991,** *74,* 469-472.
210. Talekar, N.S.; Lee, S.T. *Bull. Inst. Zoology Academia Sinica* **1989,** *28,* 281-288.
211. Heath, R.R.; Coffelt, J.A.; Proshold, F.I.; Jansson, R.K.; Sonnet, P.E. *Sweet Potato Pest Management* **1991,** 79-96.
212. Renou, M. *Bulletin de La Societe Zoologique de France - Evolution et Zoologie* **1991,** *116,* 339-344.
213. Unnithan, G.C.; Paye, S.O. *Environ. Entomol.* **1991,** *20,* 150-155.
214. Walker, P.W. *J. Aust. Entomol. Soc.* **1991,** *30,* 339-340.
215. Minks, A.K.; Carde, R.T. *Entomol. Exper. Appl.* **1988,** *49,* 25-36.

216. Niwa, C.G.; Daterman, G.E. *Environ. Entomol.* **1989,** *18,* 570-574.
217. Spittler, T.D.; Leichtweis, H.C.; Dennehy, T.J. *ACS Symp. Ser.* **1988,** *379,* 430-436.
218. Gray, D.R.; Borden, J.H. *J. Econ. Entomol.* **1989,** *82,* 1399-1405.
219. Gray, D.R.; Holsten, E.; Pascuzzo, M. *Canadian Entomologist* **1990,** *122,* 373-379.
220. Shore, T.L.; Hall, P.M.; Maher, T.F. *J. Appl. Entomol.* **1990,** *109,* 315-319.
221. Hokkanen, H.M.T. *Annu. Rev. Entomol.* **1991,** *36,* 119-138.
222. Knipling, E.F.; McGuire, J.U. *USDA Inform. Bull.* **1966,** *308,* 20.
223. Bakke, A. In *Insect Suppression with Controlled Release Pheromone Systems*; Kydonieus, A. F. Beroza, M.; CRC Press: Boca Raton, **1982**; pp 17-26.
224. Bakke, A. *Holarctic Ecol.* **1989,** *12,* 515-519.
225. Bakke, A. *Forest. Ecol. Manage.* **1991,** *39,* 299-303.
226. Quiring, D.T.; Timmins, P.R. *J. Appl. Ecol.* **1990,** *27,* 965-982.
227. Sternlicht, M.; Barzakay, I.; Tamim, M. *Entomol. Exper. Appl.* **1990,** *55,* 59-67.
228. Weslien, J.; Lindelow, A. *Can. J. Forest Res.* **1990,** *20,* 1786-1790.
229. Brenner, R.J.; Pierce, R.R. *J. Econ. Entomol.* **1991,** *84,* 1735-1745.
230. Snetsinger, R.; Sheltar, D.J. *Melshimer Entomol.* **1982,** *32,* 12-19.
231. Paul, L.C. *Can. Entomol.* **1942,** *74,* 77-78.
232. Lanier, G.N. In *Behavior-Modifying Chemicals for Insect Management - Applications of Pheromones and Other Attractants*; Ridgway, R. L.; Silverstein, R. M. Inscoe, M. N.; Marcel Dekker: New York, **1990**; pp 25-45.
233. Metcalf, R.L.; Metcalf, R.A.; Rhodes, A.M. *Proc. Natl. Acad. Sci. USA* **1980,** *77,* 3769-3772.
234. Metcalf, R.L.; Rhodes, A.M.; Metcalf, R.A.; Ferguson, J.; Metcalf, E.R.; Lu, P.-Y. *Environ. Entomol.* **1982,** *11,* 931-937.
235. Sutter, G.R.; Connelly, J.; Hesler, L.S.; Ellsbury, M.M.; Woodson, W.D. *Annual Meeting, North Central Branch of the Entomological Society of America, Kansas City, Missouri,* **1992.**
236. Lance, D.R.; Sutter, G.R. *J. Econ. Entomol.* **1990,** *83,* 1085-1090.
237. Lance, D.R.; Sutter, G.R. *J. Econ. Entomol.* **1991,** *84,* 1861-1868.
238. Butler, R.E. *Agricultural Aviation* **1992,** *6,* 8-20.
239. Williams, D.F.; Lofgren, C.S.; Vandermeer, R.K. *J. Econ. Entomol.* **1990,** *83,* 67-73.
240. Banks, W.A.; Las, A.S.; Adams, C.T.; Lofgren, C.S. *J. Entomol. Sci.* **1992,** *27,* 50-55.
241. Stein, M.B.; Thorvilson, H.G.; Johnson, J.W. *Florida Entomol.* **1990,** *73,* 117-123.
242. Gregory, D.A.; Johnson, D.L.; Thompson, B.H.; Richards, K.W. *J. Agric. Entomol.* **1992,** *9,* 109-115.
243. Quinn, M.A.; Kepner, R.L.; Swain, J.L.; Walgenbach, D.D.; Foster, R.N.; Bohls, R.A.; Reuter, K.C.; Pooler, P.D. *Can. Entomol.* **1989,** *121,* 589-602.
244. McGuire, M.R.; Streett, D.A.; Shasha, B.S. *J. Econ. Entomol.* **1991,** *84,* 1652-1656.
245. Zeman, P.; Kyselova, M.; Rupes, V.; Wimmer, Z.; Vrba, Z. *Entomol. Exper. Appl.* **1992,** *62,* 47-53.
246. Georgis, R.; Wojcik, W.F.; Shetlar, D.J. *Florida Entomol.* **1989,** *72,* 203-204.
247. Murray, D.A.H. *Crop Protection* **1989,** *8,* 397-398.
248. Barclay, H.J. *Bull. Entomol. Res.* **1988,** *78,* 573-590.
249. Steiner, L.F. *J. Econ. Entomol.* **1952,** *J. Econ. Entomol.,* 241-248.

250. Chambers, D.L. In *Chemical Control of Insect Behavior: Theory and Application*; Shorey, H. H. McKelvey, J. J.; Wiley-Interscience: New York, **1977**; pp 327-344.
251. Wakabayashi, N.; Cunningham, R.T. *J. Econ. Entomol.* **1991**, *84*, 1672-1676.
252. Butler, G.D.; Las, A.S. *J. Econ. Entomol.* **1983**, *76*, 1448-1451.
253. Beasley, C.A.; Henneberry, T.J. *Proceedings, 1983 Beltwide Cotton Production Conference, San Antonio, Texas,* **1984**.
254. Miller, E.; Staten, R.T.; Nowell, C.; Gourd, J. *J. Econ. Entomol.* **1990**, *83*, 1321-1325.
255. Landolt, P.J.; Lenczewski, B.; Heath, R.R. *J. Econ. Entomol.* **1991**, *84*, 1344-1347.
256. Phelan, P.L.; Baker, T.C. *J. Econ. Entomol.* **1987**, *80*, 779-783.
257. Trematerra, P.; Capizzi, A. *J. Appl. Entomol.* **1991**, *111*, 451-456.
258. McKibben, G.H.; Smith, J.W.; Mcgovern, W.L. *J. Entomol. Sci.* **1990**, *25*, 581-586.
259. Dedek, W.; Grimmer, F.; Pape, J.; Korner, H.J. *Forest Ecol. Manag.* **1988**, *26*, 63-76.
260. Lewis, W.J.; Martin, W.R. *J. Chem. Ecol.* **1990**, *16*, 3067-3089.
261. Powell, W.; Decker, U.M.; Budenberg, W.J. *Behaviour and Impact of Aphidophaga* **1991**, 67-71.
262. Dicke, M.; Sabelis, M.W.; Takabayashi, J.; Bruin, J.; Posthumus, M.A. *J. Chem. Ecol.* **1990**, *16*, 3091-3118.
263. Lewis, W.J.; Nordlund, D.A.; Gueldner, R.C.; Teal, P.E.A.; Tumlinson, J.H. *J. Chem. Ecol.* **1982**, *8*, 1323-1331.
264. Blaney, W.M.; Simmonds, M.S.J.; Ley, S.V.; Anderson, J.C.; Toogood, P.L. *Entomol. Exper. Appl.* **1990**, *55*, 149-160.
265. Jilani, G.; Saxena, R.C. *J. Econ. Entomol.* **1990**, *83*, 629-634.
266. Xie, Y.S.; Arnason, J.T.; Philogene, B.J.R.; Lambert, J.D.H.; Kaminski, J.; Morand, P.; Timmins, G.; Werstiuk, N.H. *Can. Entomol.* **1991**, *123*, 707-710.
267. Isman, M.B.; Koul, O.; Luczynski, A.; Kaminski, J. *J. Agric. Food Chem.* **1990**, *38*, 1406-1411.
268. Bentley, M.D.; Alford, A.R.; Mendel, M.J.; Hassanali, A.; Rajab, M.S. *Entomol. Exper. Appl.* **1988**, *49*, 189-193.
269. Serit, M.; Ishida, M.; Hagiwara, N.; Kim, M.; Yamamoto, T.; Takahashi, S. *J. Chem. Ecol.* **1992**, *18*, 593-603.
270. Nawrot, J.; Harmatha, J.; Kostova, I.; Ognyanov, I. *Biochem. Syst. Ecol.* **1989**, *17*, 55-57.
271. Chan, T.H.; Guertin, K.R.; Prasad, C.V.C.; Thomas, A.W.; Strunz, G.M.; Salonius, A. *Can. J. Chem.* **1990**, *68*, 1170-1177.
272. Griffiths, D.C.; Woodcock, C.M.; Pickett, J.A.; Smart, L.E. *Pesticide Sci.* **1989**, *27*, 269-276.
273. Bloszyk, E.; Nawrot, J.; Harmatha, J.; Drozdz, B.; Chmielewicz, Z. *J. Appl. Entomol.* **1990**, *110*, 96-100.
274. Mohan, D. *Curr. Sci.* **1990**, *59*, 955-955.
275. Prasad, M.P.; Kalyanasundaram, M. *Indian J. Med. Res. Section A* **1990**, *91*, 453-457.
276. Reifenrath, W.G.; Kurtz, M.S.; Hawkins, G.S. *J. Amer. Mosquito Control Assoc.* **1989**, *5*, 45-51.
277. Schreck, C.E.; Mcgovern, T.P. *J. Amer. Mosquito Control Assoc.* **1989**, *5*, 247-250.

278. Schreck, C.E.; Kline, D.L. *J. Amer. Mosquito Control Assoc.* **1989,** *5,* 77-80.
279. Schreck, C.E.; Kline, D.L. *J. Amer. Mosquito Control Assoc.* **1989,** *5,* 91-94.
280. Annis, B. *J. Amer. Mosquito Control Assoc.* **1991,** *7,* 543-546.
281. Curtis, C.F.; Hill, N. *Entomol. Exper. Appl.* **1988,** *49,* 175-179.
282. Gupta, R.K.; Rutledge, L.C. *J. Amer. Mosquito Control Assoc.* **1989,** *5,* 52-55.
283. Mani, T.R.; Reuben, R.; Akiyama, J. *J. Amer. Mosquito Control Assoc.* **1991,** *7,* 565-568.
284. Rutledge, L.C. *J. Amer. Mosquito Control Assoc.* **1988,** *4,* 414-425.
285. Steltenkamp, R.J.; Hamilton, R.L.; Cooper, R.A.; Schal, C. *J. Med. Entomol.* **1992,** *29,* 141-149.
286. Veer, V.; Prasad, R.; Rao, K.M. *Text. Res. J.* **1991,** *61,* 655-659.
287. Robert, L.L.; Coleman, R.E.; Lapointe, D.A.; Martin, P.J.S.; Kelly, R.; Edman, J.D. *J. Med. Entomol.* **1992,** *29,* 267-272.
288. Kumar, S.; Prakash, S.; Kaushik, M.P.; Rao, K.M. *Med. Vet. Entomol.* **1992,** *6,* 47-50.
289. Blum, M.S.; Jones, T.H.; Fales, H.M. *Abstr. Papers Amer. Chem. Soc.* **1989,** *198,* 79-.
290. Dube, S.; Upadhyay, P.D.; Tripathi, S.C. *Can. J. Bot.* **1989,** *67,* 2085-2087.
291. Singh, D.; Singh, A.K. *Insect Science and Its Application* **1991,** *12,* 487-491.
292. Durairaj, C.; Soorianathasundaram, K.; Nambisan, K.M.P. *Indian J. Agric. Sci.* **1991,** *61,* 959-960.
293. Kwan, S.; Sporns, P. *J. Apicultural Res.* **1988,** *27,* 162-168.
294. Larew, H.G.; Locke, J.C. *Hortscience* **1990,** *25,* 1406-1407.

RECEIVED October 6, 1992

Chapter 15

Insecticidal Pyrroles

D. G. Kuhn, R. W. Addor, R. E. Diehl, J. A. Furch, V. M. Kamhi, K. E. Henegar, K. A. Kremer, G. T. Lowen, B. C. Black, T. P. Miller, and M. F. Treacy

**Agricultural Research Division, American Cyanamid Company,
P.O. Box 400, Princeton, NJ 08543–0400**

The development of a new series of insecticidal pyrroles, based on the natural product dioxapyrrolomycin, is described. In particular, new chemistry leading to halogenated and trifluoromethyl-containing pyrroles is presented. This new class of insect control agents has been shown to be active on a broad spectrum of insect pests. It is proposed that the mode of action for this series is the uncoupling of oxidative phosphorylation. One member of this series, AC 303,630 [4-bromo-2-(4-chlorophenyl)-1-(ethoxymethyl)-5-(trifluoromethyl)pyrrole-3-carbonitrile] is currently undergoing development as a broad spectrum insecticide/miticide.

The discovery of new strategies, whether chemical or biological, for the control of insect pests remains one of the premier challenges facing scientists today. A variety of approaches to lead generation and optimization of biological activity has been developed. In particular, the world of natural products has been the starting point for many compounds with insecticidal activity. This chapter details our effort to develop a new, effective insect control agent based on a naturally occurring compound.

The genesis of this work was our screening group's discovery of the insecticidal activity of a fermentation broth from a Streptomyces strain. The active component was isolated and identified by our scientists at our Lederle Laboratories and was shown to have the structure in Figure 1. It has been named dioxapyrrolomycin (*1*).

At about the same time, the identical pyrrole was reported by Mëiji Seika and SS Pharmaceutical Company in Japan to have activity against Gram-positive and some Gram-negative bacteria (*2, 3*). Neither group reported insecticidal activity. While dioxapyrrolomycin had moderate, broad spectrum insecticidal activity as shown in Figure 1, the compound was found to be highly toxic to mice (oral LD_{50} of about 14 mg/kg). This combination preculed the natural product as a candidate for development. However, the simplicity of the structure suggested this compound as a starting point for synthetic modification (*4, 5*).

0097–6156/93/0524–0219$06.00/0

Synthesis

We chose the left portion of the dioxapyrrolomycin molecule, i.e., the dihalonitropyrrole, as our starting point, thereby eliminating the methylenedioxy subunit. At the outset of this work, we found the need to develop synthetic methodology suitable to highly functionalized pyrroles.

2-Aryl-3-cyano/nitro-4,5-dihalo Pyrroles. Our initial efforts required an efficient synthesis of the dihalo pyrrole nucleus that allowed for variation of substituents on the aryl ring and also the ability to change the electron withdrawing group at the 3-position. The synthesis that was developed is shown in Figure 2. Condensation of the ketones (**1**) with aminoacetaldehyde diethylacetal gave the enamines (**2**) as a mixture of isomers. Treatment with trifluoroacetic acid gave the 2-aryl-3-cyano/nitro pyrroles (**3**) in good yield. Halogenation using bromine or sulfuryl chloride gave the desired 4,5-dibromo or 4,5-dichloropyrroles (**4**).

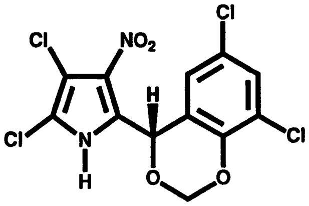
2-Aryl-5-trifluromethyl Pyrrole Derivatives. Synthesis efforts have been directed toward replacing the halogen substituents at the 4 and/or 5 positions of the molecule. A particularly intriguing candidate is the trifluoromethyl (CF_3) group. This group, considered a "pseudo-halogen", often imparts interesting biological activity when introduced into molecules (*6*). However, methods for the direct introduction of the CF_3 group onto deactivated aromatic systems were scarce. Therefore, it was necessary to develop new procedures that would allow for the pyrrole to be formed with the CF_3 group in place. This was accomplished using cycloaddition chemistry (Figure 3). Although 1,3-dipolar cycloadditions have been utilized extensively to prepare heterocycles (*7*), these methods were not used to prepare pyrroles substituted with a trifluoromethyl group.

In this procedure, an oxazolinone (**6**) derived from a suitably substituted phenylglycine (**5**), reacts with an olefin bearing an electron-withdrawing group and a leaving group such as a halogen (**7**) in the presence of a base to give the 2-aryl-5-trifluoromethylpyrrole (**8**) in good to excellent yield (*5*). This reaction is rapid, usually requiring less than one hour, and occurs under mild conditions. It has been found that the reaction is regiospecific, and yields only the isomer shown.

If R^1= H, in the olefin (**7**) the 4-unsubstituted pyrrole is produced. Treatment of (**8**) (R^1= H) with bromine/sodium acetate/acetic acid cleanly introduces bromine into the 4-position to give (**9**). In a similar manner, using an olefin substituted with a trifluoromethyl group (**7**) (R^1= CF_3) gives the desired 4,5-*bis*-trifluoromethylpyrrole (**8**) (R^1= CF_3) analog of the lead 4,5-dihalopyrroles. By using various substituted phenylglycines available *via* the Strecker synthesis, a series of mono- or *bis*-trifluoromethylsubstituted pyrroles has been prepared.

A special case of this chemistry has been used as a key step in the preparation of the 2-aryl-3-nitro-5-trifluoromethylpyrroles (Figure 4). Cycloaddition of the oxazolinone (**6**) (R= 4-Cl) with the pyridinium salt (**10**) in pyridine gave the 2-aryl-5-trifluoromethyl pyrrole (**11**) in good yield. In this reaction, the pyridine acts as both an activating group for the olefin and also as the leaving group. Nitration of (**11**) gave a separable mixture of the 3- and 3,4-dinitropyrroles of which the 3-nitro isomer (**12**) predominates. Bromination of (**12**) gives the desired 2-aryl-3-nitro-4-bromo-5-trifluoromethylpyrrole (**13**).

During the course of our work aimed at optimizing the insecticidal activity, we encountered an unexpected drawback. Certain of these compounds were highly phytotoxic. In an attempt to overcome this deficiency and to see if other benefits might accrue, the effect of N-derivatization of these pyrroles was studied (Figure 5). Alkylation of the parent pyrroles (**8**) produced the N-protected compounds (**14**). A number of N-derivatized pyrroles were found to retain the high biological activity of the parent with little or no observed phytotoxicity.


Cl
NO2
Cl
H
Cl
Cl
N
H
O
O

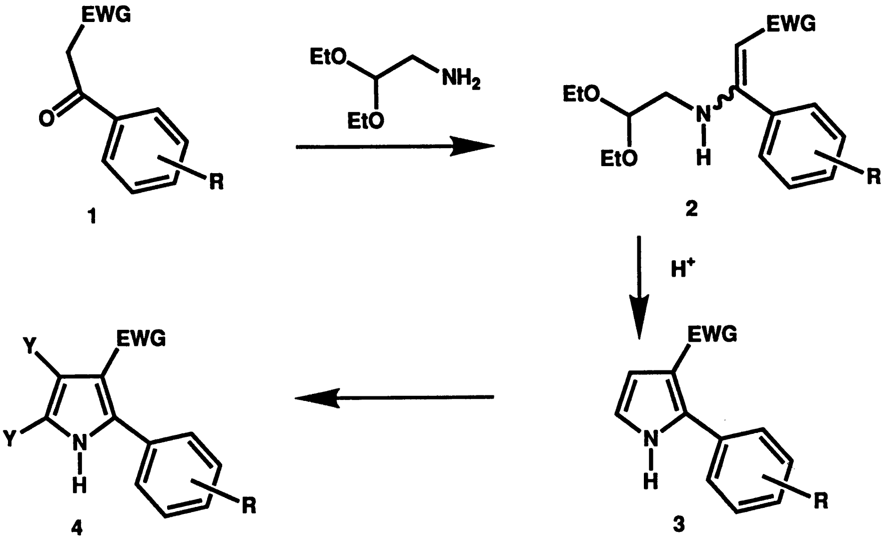
Dioxapyrrolomycin

Insecticidal Activity

LC_{50} (ppm)

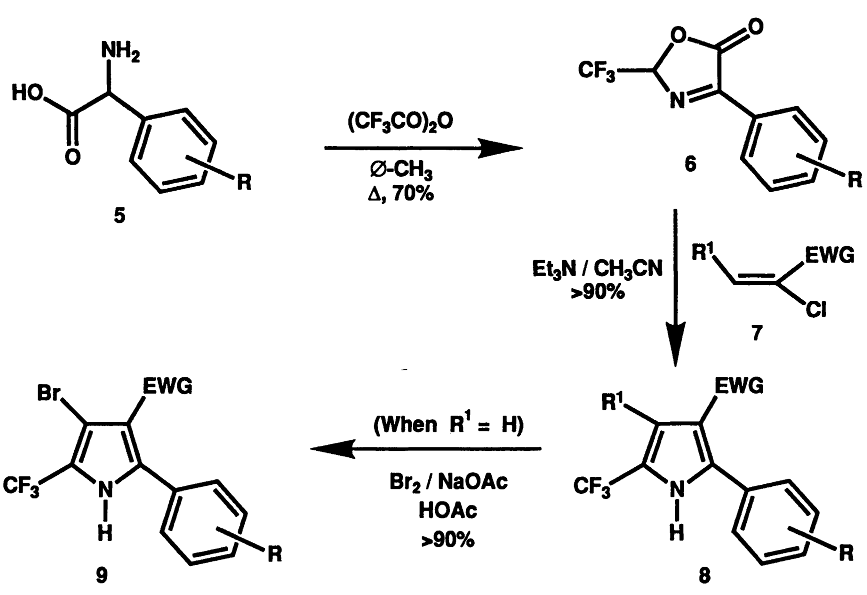
Southern Armyworm *Spodoptera eridania* **3rd Instar**	**Tobacco Budworm** *Helothis virescens* **3rd Instar**	**2-Spotted Mite** *Tetranychus urticae* **P-Resistant**	**Western Potato Leafhopper** *Empoassa abrypta* **Mixed**
40	32	10	<100

Figure 1. Structure and Insecticidal Properties of Dioxapyrrolomycin.


EWG
O
R
1
EtO
NH2
EtO
EWG
EtO
N
H
EtO
R
2
H+
EWG
N
H
R
3
Y
EWG
Y
N
H
R
4

Where EWG = CN, NO_2
Y = Cl, Br

Figure 2. Synthesis of 2-Aryl-4,5-dihalopyrrole Derivatives.


NH2
HO
O
R
5
(CF3CO)2O
Ø-CH3
Δ, 70%
CF3
O
O
N
6
R
Et3N / CH3CN
>90%
R1
EWG
Cl
7
Br
EWG
CF3
N
H
R
9
(When R1 = H)
Br2 / NaOAc
HOAc
>90%
R1
EWG
CF3
N
H
R
8

Where EWG = CN, CO_2R^2 and COR^2

Figure 3. Synthesis of 5-CF_3 Pyrrole Derivatives.

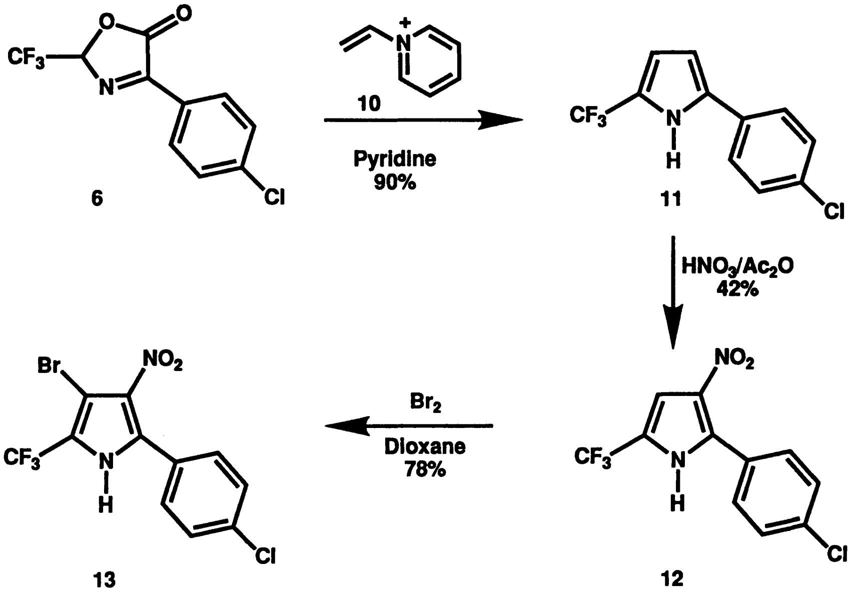

CF3
O
O
N
6
Cl
+
N
10
Pyridine
90%
CF3
N
H
11
Cl
HNO3/Ac2O
42%
Br
NO2
CF3
N
H
Cl
13
Br2
Dioxane
78%
NO2
CF3
N
H
Cl
12

Figure 4. Synthesis of 2-Aryl-3-NO_2-5-CF_3 Pyrrole Derivatives.

Insecticidal Activity

All compounds prepared in this study were screened against third instar southern armyworms (SAW), *Spodoptera eridania* (Cramer), first and third instar tobacco budworms (TBW) *Heliothis virescens* (F) and western potato leafhopper (WPL) *Empoasia abrupta* (De Long), using a standard leaf dip feeding bioassay with technical material. When possible, LC_{50} values were determined and reported in parts per million (ppm).

2-Aryl-3-cyano/nitro-4,5-dihalo Pyrroles. Figure 6 shows the insecticidal activity of a select group of 4,5-dichloropyrroles against southern armyworms and tobacco budworms. In this series, we have varied the substituents on the phenyl ring at the 2-position of the pyrrole. The activity of both the 3-cyano and the 3-nitro analogs is presented.

The insecticidal activity of the natural product, dioxapyrrolomycin (Dioxa) is included for comparison. Against SAW, both the 3-cyano and the 3-nitro compounds were significantly more active than dioxapyrrolomycin when the substituent was halogen (**15**, **16**) or CF_3 (**17**). The 3-cyano isomers achieved a level of activity superior to dioxapyrrolomycin when tested against tobacco budworms. However, the 3-nitro analogs were less effective in controlling this species.

Figure 7 compares the activity of a series of 2-(*p*-chlorophenyl)-3-cyano pyrroles in which the groups at the 4 and 5 position of the pyrrole ring have been varied. All of the compounds showed good activity against SAW.

The 4,5-dichloro (**15**) and 4,5-dibromo (**18**) pyrroles were roughly equivalent against the TBW. It should be noted that two halogens are required; mono-halogenated pyrroles were far less active. Moreover, a dramatic increase in potency was observed when the halogen at the 5-position was replaced with a trifluoromethyl group. The *bis* trifluoromethyl compound (**21**) retained activity against the first instar TBW but was less effective when tested on third instar TBW.

2-Aryl-5-trifluoromethylpyrrole-3-carbonitriles. With the discovery of improved activity of 2-(*p*-chlorophenyl)-4-bromo-5-trifluoromethylpyrrole-3-carbonitrile, a series of phenyl substituted analogs was prepared. Some of the results are given in Table I.

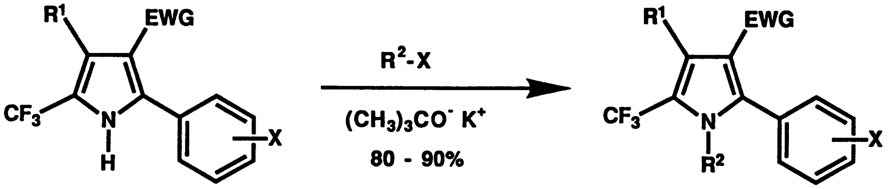
The compounds with halogen, (**19**, **23-24**) trifluoromethyl (**25**), or dihalo (**31**) substitution effectively controlled lepidopterous insects and, in some cases, WPL. However, substitution with methyl (**26**), methoxy (**27**), or hydroxyl (**28**) groups produces inactive compounds while nitro substitution (**29**) gives reduced control of lepidopterous insects and no activity in WPL.

N-Derivatization. The phytotoxicity observed for some of the compounds prepared in this work led to a study of various blocking groups on the nitrogen in an effort to suppress the undesired side effect while maintaining insecticidal potency. Some of the results are summarized in Figure 8.

N-Methylation of the parent compound (**19**) (R= H to R= CH_3) results in a reduction of activity.

The N-ethoxymethyl derivative (R= CH_2OEt) (**33**) retained the high insecticidal activity of the parent pyrrole (R= H) (**19**) while at the same time showing no phytotoxicity. Other alkoxyalkyl derivatives also showed good activity against the target species and little or no phytotoxicity. The N-cyano derivative (R= CN) (**36**) was also highly active.

A cyano (or to a lesser extent, nitro) group at the 3 position of the pyrrole


R1
EWG
CF3
N
H
X
R2-X
(CH3)3CO- K+
80 - 90%
R1
EWG
CF3
N
R2
X

8

14

Figure 5. N-Derivatization of Pyrroles.

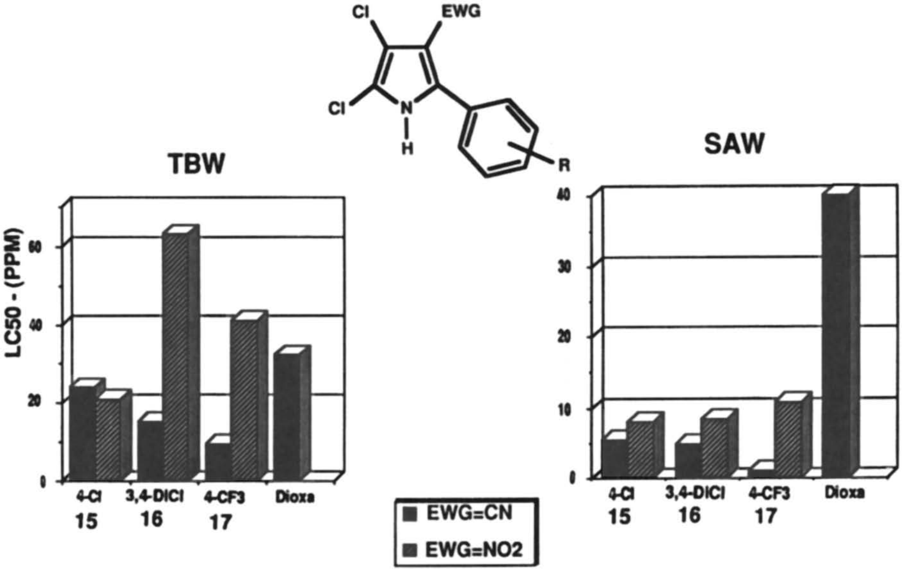


Figure 6. Insecticidal Activity of 4,5-Dihalopyrroles.

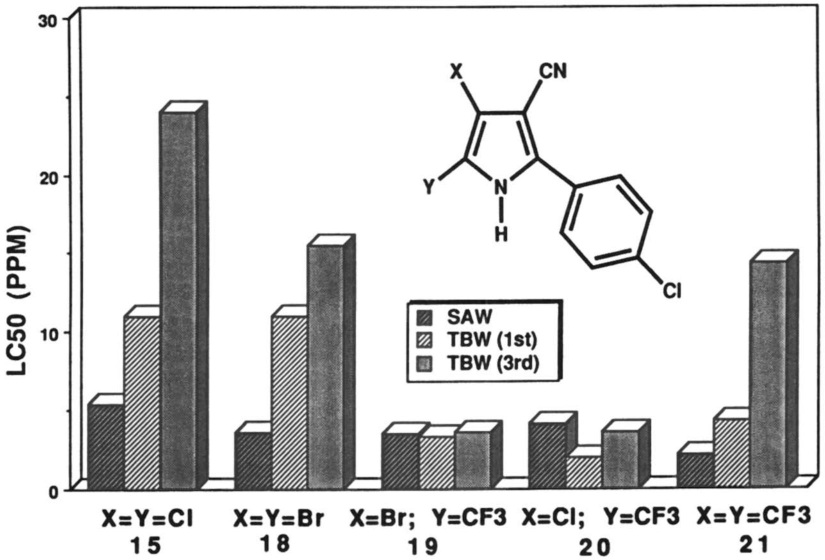
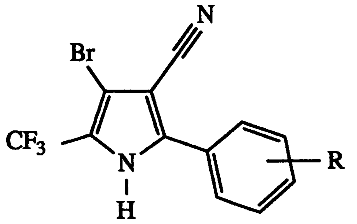


Figure 7. Insecticidal Activity of 4,5-Disubstituted Pyrrole-3-carbonitriles.

Table I. Insecticidal Activity of 2-Aryl-5-CF$_3$-pyrrole-3-carbonitriles



		% Control at 100 ppm		
		Southern Armyworm *S. eridania*	Tobacco Budworm *H. virescens*	Western Potato Leafhopper *E. abrupta*
	R	3rd instar	3rd instar	
22	H	100	100	0
19	4-Cl	100	100	100
23	4-F	100	100	90
24	4-Br	100	100	100
25	4-CF$_3$	100	100	100
26	4-CH$_3$	100	0	0
27	4-OCH$_3$	0	0	0
28	4-OH	0	0	0
29	4-NO$_3$	80	80	0
30	4-Cl	100	100	100
31	3,4-Cl$_2$	100	100	100

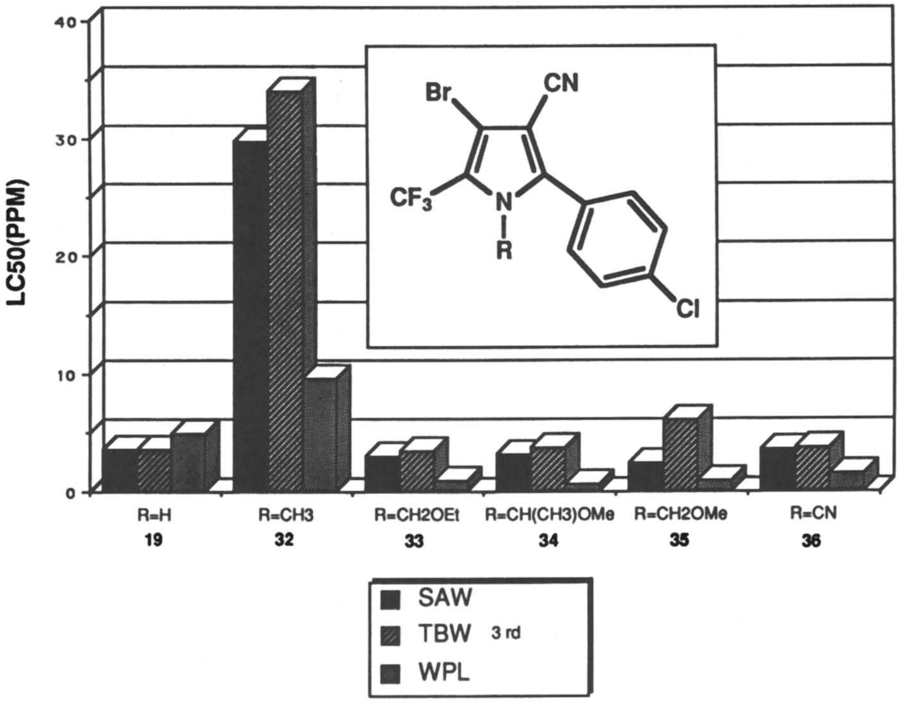
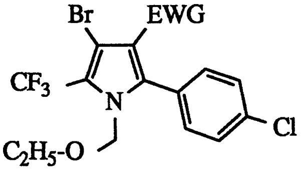


Figure 8. Insecticidal Activity of N-Derivatized Pyrroles.

ring provided high activity. A study was conducted to determine whether other electron withdrawing groups would be as effective as cyano or nitro (Table II).

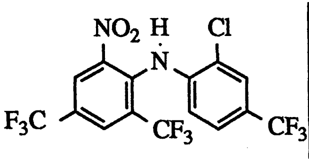
Table II. Insecticidal Activity of N-Derivatized 3-Substituted Pyrroles



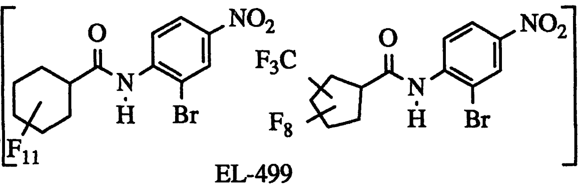
	% Control at 100 ppm		
	SAW *S. eridania* 3rd instar	TBW *H. virescens* 3rd instar	Western Potato Leafhopper *E. abrupta*
EWG			
33 CN (AC 303630)	100	100	100
37 CO_2H_3	0	0	0
38 $COCH_3$	60	0	0
39 $CONH_2$	0	0	0
40 CH≡C	0	0	0

At 100 ppm, the 3-cyano derivative (33), designated AC 303,630, gave complete control of SAW, third-instar TBW, and WPL. Replacement of the cyano group with the methyl ester (37), amide (39) or the alkyne (40) resulted in complete loss of activity. Only the methyl ketone (38) gave partial control of SAW at this level.

Mode of action Studies. The interference with respiration and, in particular, the uncoupling of oxidative phosphorylation has been proposed as the mode of action for a number of pesticides (*8, 9*). For example, the acaracide fentrifanil (*10, 11*) and, the soil insecticide EL-499 (*12*) have been shown to be potent uncouplers of oxidative phosphorylation.



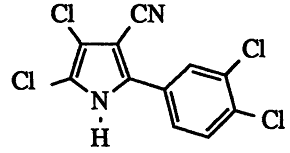
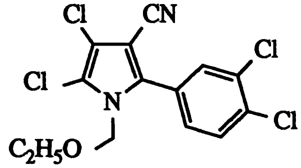
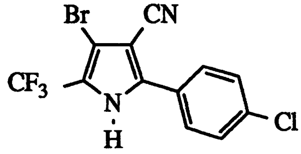
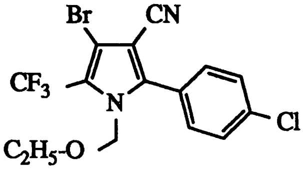
Fentrifanil



EL-499

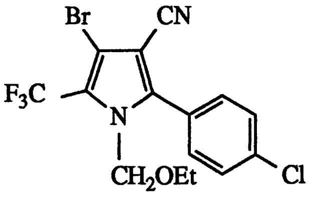
Certain physiochemical features of the pyrroles such as lipophicity and acidity suggested that these compounds were also acting as uncouplers. This was verified by oxygen electrode experiments using rat liver mitochondria (Table III).

Table III. Comparison of in vitro and in vivo Toxicities of Selected Pyrroles

		Mitochondria UC_{50}(nM)	SAW LC_{50}(ppm)
16		2.0	4.7
41		150.0	2.5
19		2.4	3.5
33		1000	3.0

In the mitochondrial assay, the dihalopyrrole (**16**) and the trifluoromethyl pyrrole (**19**) were both potent uncouplers causing 50% uncoupling at 2 nM, and 2.4 nM, respectively. The N-ethoxymethyl derivatives were virtually inactive in this assay. However, *in vivo* the compounds (**33** and **41**) were as active as the parent compound against southern armyworms. These differences between *in vivo* and *in vitro* activity suggest that the N-derivatized pyrroles are acting as "pro-insecticides" and, that after metabolic removal of the group on nitrogen, the parent pyrrole is functioning as the uncoupler. This is in agreement with the suggestion that effective uncouplers are both lipophilic and have pKa's in the range of 4.5-6.2 (*13*). The pKa of the pyrrole (**19**) is 5.6 while calculated logP is 5.6.

AC 303,630. Table IV summarizes some of the structure-activity trends that have emerged from this study. Based on these observations, the compound that has been selected for development is AC 303,630 (**33**). A comparison of the toxicities of AC 303,630 and the starting lead dioxapyrrolomycin, is presented in Figure 9.

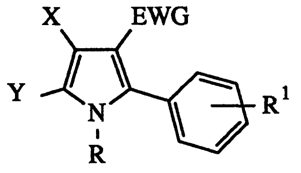


AC 303,630 (**33**)

It has therefore been possible, though synthetic manipulation, to optimize the insecticidal activity while reducing the mammalian oral toxicity. In addition, AC 303,630 has low dermal toxicity i.e. rabbit dermal LD_{50}= >2000 mg/kg and a rat oral LD_{50} of 662 mg/kg. This combination of properties has led to the large scale development of AC 303,630 (**33**) towards introduction as a commercial product. A comparison of AC 303,630 vs. some commercial standards on third instar TBW is shown in Figure 10 (*14*).

A series of studies has been undertaken to evaluate the potential of AC 303,630 in IPM programs. The preliminary results of one of these tests are shown in Figure 11. In this work, the toxicity of an EC formulation of AC 303,630 on a susceptible strain of pest mite (*Tetranychus urticae* Koch) and on a predatory mite (*Metaseiulus occidentalis*) was determined. It was found that AC 303,630 had low toxicity toward the predatory species but was highly active on the pest mites. Further evaluation of AC 303,630 (**33**)against other predatory species along with additional field studies is underway.

Table IV. Structure-activity Trends in the Pyrrole Series



1. X and Y must be Cl, Br, or CF_3 for maximum activity.
2. EWG = CN or NO_2 pproximately equivalent.
3. R^1 requires some electron withdrawal (Cl, Br, or CF_3 are best).
4. Best activity when R^1substituent is in the 4-position. Also 3,4-disubstituted compounds are fairly good.
5. R= alkoxyalkyl produces compounds with high insecticidal activity an reduced phytotoxicity relative to the parent.
6. N.Derivatized pyrroles appear to be acting as pro-insecticides with in vivo removal of the N-protecting group necessary for high uncoupling activity.

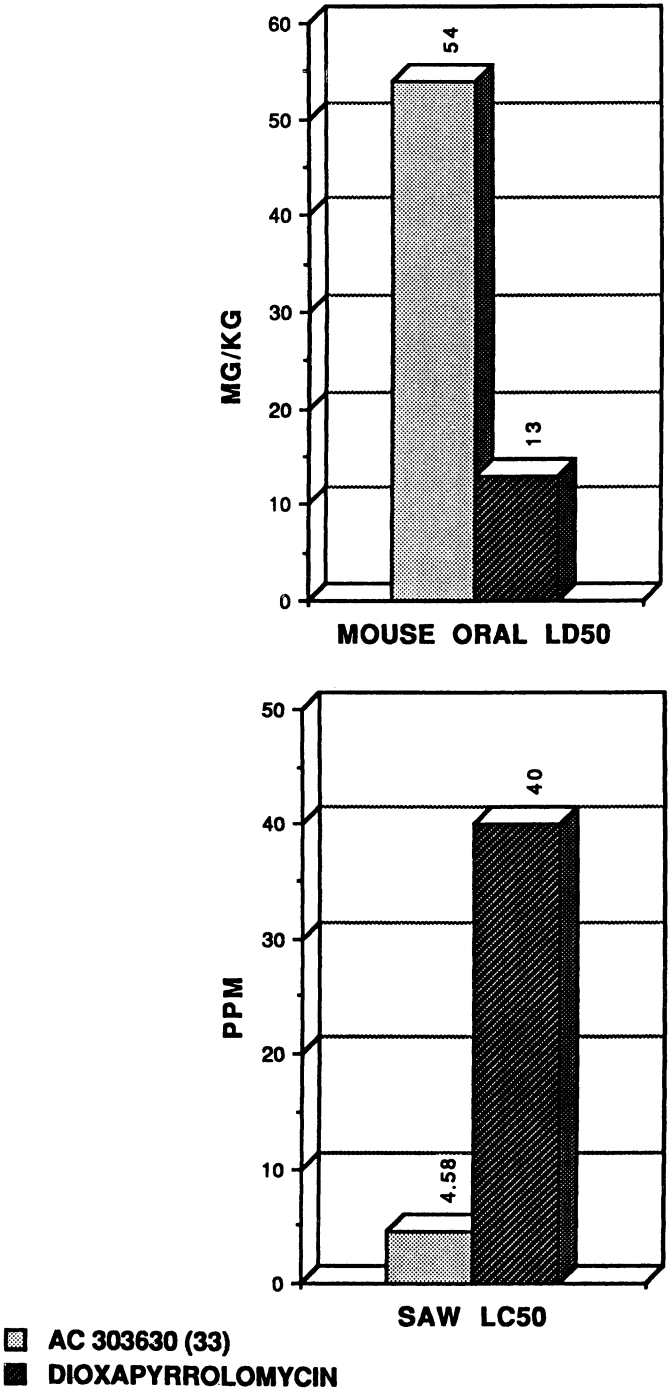


Figure 9. Comparative Toxicities of AC 303,630 (33) and Dioxapyrrolomycin.

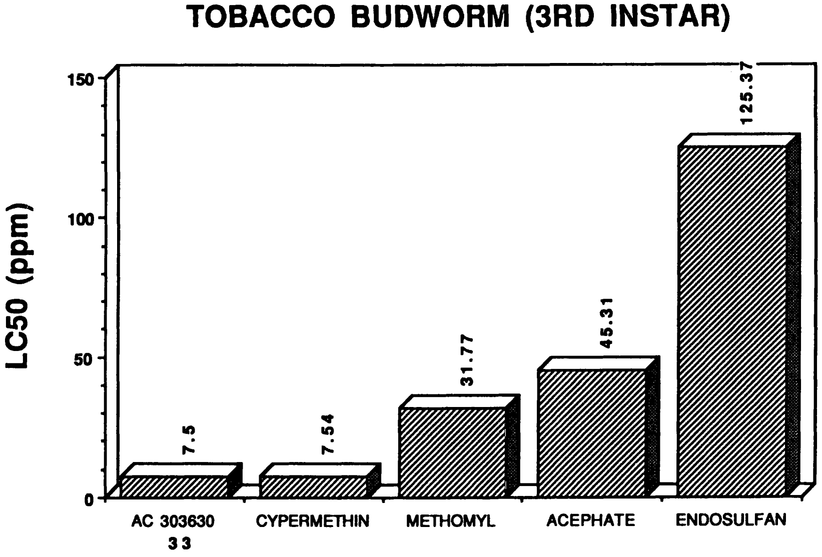


Figure 10. Comparison of the Toxicities of Selected Insecticides.

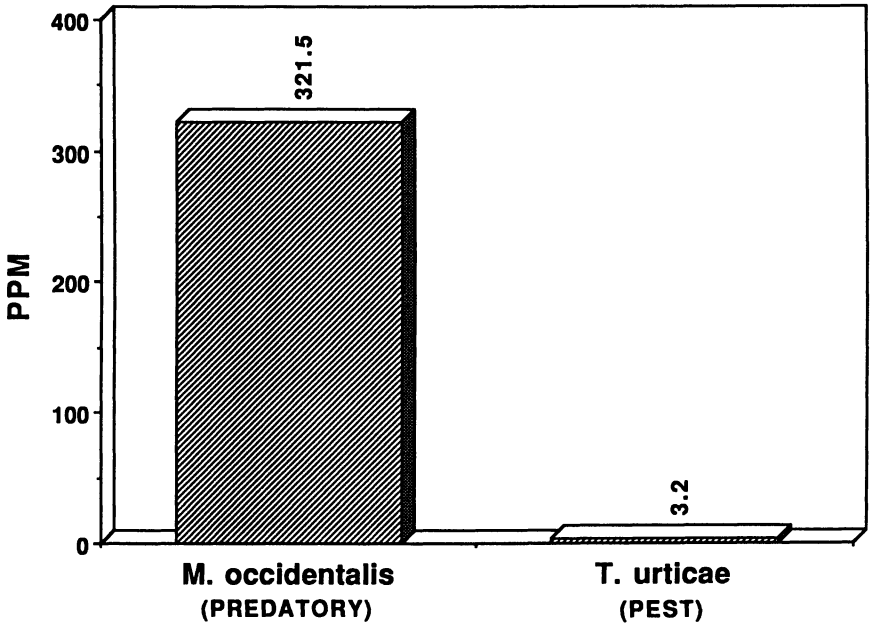


Figure 11. LC_{50} Values of AC 303,630 (33) on Mites.

Acknowledgements

The authors wish to thank M. Brandolino, D. Wright, Jr, J. Lovell, R. Borysewicz, C. Gronostajski, and M. Rivera for their assistance in obtaining the biological data. The help of D. Gange in defining activity relationships based on acidities and partition coefficients is also gratefully acknowledged.

Literature Cited

1. Carter, G; Nietsche, J; Goodman, J.; Torray, M.; Dunne, T.; Borders, D.; Testa, R. *J. Antibiotics* **1987**, *40*, 233,
2. Nakamura, H.; Shiomi, K.; Iinuma, H.; Naganawa, H.; Obata, T.; Takeuchi, T.; Umezawa, H. *J. Antibiotics* **1987**, *40*, 899.
3. Yano, K.; Oono, J.; Magi, K.; Asaoka, T.; Nakashima, T. *J. Antibiotics* **1987**, *40*, 961.
4. Addor, R.; Brow, D.; Babcock, T.; Diehl, R.; Furch, J.; Kameswaran, V.; Kamhi, V; Kremer, K; Kuhn, D.; Lovell, J.; Lowen, G.; Miller, T.; Peevy, R.; Siddens, J.; Treacy, M.; Trotto, S.; Wright, D.; Presented in part at the 201st ACS National Meeting, Atlanta, GA, April, 1991.
5. Kuhn, D.; Kamhi, V.; Furch, J; Diehl, R.; Trotto, S.; Lowen, G.; Babcock, T.; Presented in part at the 201st ACS National Meeting, Atlanta, GA, April, 1992.
6. Filler, R.; Kobayashi, Y.; "Biomedical Aspects of Fluorine Chemistry", Elsevier Biomedical Press, New York, N. Y. **1986**.
7. Padwa, A., ed., "1,3-Dipolar Cycloadditon Chemistry", J. Wiley & Sons, New York, N. Y., **1984**.
8. Fukami, J.-I. In *Approaches to New Leads for Insecticides;* von Keyserlingh, H.; Jöger, A.; von Szczepanski, Ch. Eds; Springer-Verlag: Berlin, **1985**; pp 47-69.
9. *The Biochemical Mode of Action of Pesticides,* Corbett, J.; Wright, K.; Baillie, A. Eds; Academic Press: Orlando, Fl., **1984**; pp 1-49.
10. Morton, N. *Proc, 9th British Crop Protection Conference-Pests and Diseases,* **1977**, *2*, 349.
11. Nizamani, S.; Hollingworth, R. *Biochem and Biophys. Res. Comm.* **1980**, *96*, 704.
12. Gajwski, R.; Thompson, G.; Chio, E.; Alt, C.; Berard, D.; Glass, S.; Kennedy, J.; Robey, R.; van Lier, R. In *Synthesis and Chemistry of Agrochemicals II*; Baker, D.; Fenyen, J.; Moberg, W., Eds; ACS Symposium Series No. 443; American Chemical Society: Washington, DC, **1991**; pp 448-458.
13. Heytler, P. *Pharmacol. Ther.* **1980**, *10*, 461.
14. Treacy, M.; Miller, T.; Gard, I.; Lovell, J.; Wright, D.; *Proc. Beltwide Cotton Conference*, **1991**, *2*, 738.

RECEIVED August 18, 1992

Chapter 16

Arthropod-Derived Neurotoxic Insecticides

A Lead in Pesticide Science?

T. Piek

**Department of Pharmacology, University of Amsterdam, Meibergdreef 15,
1105 AZ Amsterdam, Netherlands**

Many hymenopteran insects incapacitate their prey by means of a neurotoxin. The glutamatergic excitatory neuromuscular transmission and the nicotinic excitatory synaptic transmission of insects is reversibly blocked by philanthotoxins, as well as by structurally related polyamine toxins isolated from spider venoms. These polyamine toxins are cation channel blockers or change at least the glutamatergic channel kinetics. They therefore are not receptor specific and are, for example, also active at the nicotinic receptors in the insect brain, albeit at higher concentrations. Poneratoxin, a 25 amino acid residue polypeptide isolated from an ant venom, is the first described hymenopteran neurotoxin affecting excitability of nerve and muscle fibres by changing the kinetics of the voltage-dependent sodium channel. The nicotinic synaptic transmission in the insect central nervous system (CNS) is presynaptically and irreversibly blocked by kinins, which probably cause transmitter depletion. Besides this delayed effect the carbohydrate-containing vespulakinins also show a direct and reversible inhibition of the synaptic transmission. Criticism on the possibilities to use venom toxins as leads in pesticide science has been, that these compounds have to be injected into the body or the CNS. For primary synthesis products the incorporation of the genetic codes into entomophilic viruses could solve this problem.

Several groups of hymenopteran insects, like wasps and ants, sting their prey to a paralysis or only to a behaviourial inactivation, in order to offer the prey to the own offspring as an incapacitated, but living fresh source of food. These insects have developed venoms containing natural insecticides. The ideas of the 1980's included the possibility of using natural neurotoxins from arthropods as leads to new pesticides (*4, 41-43*).

0097–6156/93/0524–0233$06.00/0

The Reversible Non-competitive Block of Synaptic Transmission by Polyamine Toxins

In the 1960's investigations started in our laboratories on the paralysing venom of the European solitary sphecid wasp *Philanthus triangulum*. The excitatory glutamatergic neuromuscular transmission of insects is antagonized by the venom and by its most active toxin (philanthotoxin 4.3.3, Figure 1) through two different effects. Firstly by a presynaptic inhibition of the high affinity glutamate uptake (*1-4*) and secondly by a block of open ion channels of the glutamate receptor-ionophore complex (*4,5*). The same toxin was isolated from the venom of an African subspecies of this wasp: *P.t. abdelcader (7)*.

Structure-activity relationship studies showed that the potency as a channel blocker of philanthotoxin depends on the presence of an aromatic moiety (*8-11*). Attempts to change the molecule into a more potent antagonist resulted in an increase in potency with factors varying from 10-100 (*8, 10-13, 22*). The pre- and postsynaptic inhibiting properties of the natural philanthotoxin were dissociated into two different analogues (*14*). Comparable polyamine-like structures have been isolated from spider venoms (*15-17*).

These polyamine toxins are also active at the mammalian CNS glutamate synaptic transmission (*18-20*) and the vertebrate and insect nicotinic transmission (*21-23*). The most active philanthotoxin analogue in the mammalian CNS is dideaza-philanthotoxin-12 *(19, 20)*.

Irreversible Depletion of Cholinergic Nerve Endings by Kinins

In 1954 Schachter and colleagues discovered a pain-producing substance in the venom of the social wasp *Paravespula vulgaris* (*24*). Due to the similarity in pharmacological properties of this substance and the bradykinin (Figure 2) they called this substance wasp kinin (*25*). The first wasp kinin, which was chemically characterized is polisteskinin 3, an octapeptide isolated from a mixture of *Polistes* species (*26*). During the following twenty years a number of kinins were isolated from social vespid wasps, like hornet, (*Vespa*), yellow jackets (*Paravespula*), and paper wasps (*Polistes*) (*27*), from solitary wasps *Megascolia, Colpa (28-30) Tiphia and Dasymutilla* (*31*), and from several ant species (*31*). They were pharmacologically characterized (*31*) and in some cases the amino acid sequence was determined (*27, 30, 33*).

It was not before the discovery of threonine-6-bradykinin (Thr^6BK) in the venom of *Megascolia flavifrons* (*28, 29*) that kinins were considered to be neurotoxins. Scoliid wasps sting into the ganglia of their prey, resulting in an irreversible paralysis. Therefore, the action of the venom and of Thr^6BK was studied using the technique of microperfusion of the sixth abdominal ganglion of the cockroach, *Periplaneta americana (28)*. At concentrations from 10^{-5}M to 10^{-4}M Thr^6BK causes an irreversible block of synaptic transmission from the cercal nerve XI to a giant interneuron. The velocity of inhibition is use (frequency) dependent like the antagonism of hemicholinium-3 (*32*).

During the 1960's Pisano and colleagues isolated, from the venom of the most offensive wasp in the United States: *Paravespula maculifrons*, a heptadecapeptide called vespulakinin 1. This kinin is highly basic and contains the nonapeptide bradykinin at the carboxyterminus (Figure 2). Most interesting is the presence of carbohydrate moieties connected to threonine residues (*33*). Synthesis of some mono- and di-glycosylated analogues of the polypeptide called "carbohydrate free" vespulakinin 1 (Figure 3) were described, (*34-36*). Recently the effects of these synthetic vespulakinin analogues on the synaptic transmission of the insect CNS were studied. Two distinctly different effects were found: a direct reversible block of excitatory nicotinic transmission and a delayed irreversible block of transmission as has been described earlier for threonine-6-bradykinin (*32*).

Poneratoxin Induces an Interconversion between Two Gating Modes of Sodium Channels

Evenomation by the ponerine ant *Paraponera clavata* causes pain and uncontrollable trembling (*37*). Recently, three neurotoxin principles, which block the synaptic transmission from the cercal excitatory nerve to a giant interneuron in the sixth abdominal ganglion of the cockroach have been separated from this venom (*38,39*). One of the principles was pharmacologically characterized as a kinin. Another and also the most active neurotoxic fraction was rechromatographed, resulting in the purification of a peptide of 25 amino acid residues, called poneratoxin (PoTX, Figure 4).

At concentrations varying from 10^{-8}M to 10^{-6}M synthetic PoTX is a strong, but very slowly acting agonist of smooth muscles, it also blocks synaptic transmission in the insect CNS in a concentration dependent manner and it depolarizes giant interneurons (*40*).

PoTX induces a concentration dependent (10^{-9}M to 5 x 10^{-6}M) prolongation of action potentials and at saturating concentration, a slow repetitive activity, developing at negative potentials (*40*). PoTX specifically acts on voltage-dependent sodium-ion channels by decreasing the peak sodium current (I_{Na}) and by simultaneously inducing a slow I_{Na} which starts to activate at -85 mV and inactivates very slowly. Both the fast and the slow components of I_{Na} are suppressed by tetrodotoxin and reverse at equal potentials, corresponding to the equilibrium potential for sodium ions. The fast component of I_{Na} has a voltage-dependence, activation and steady-state inactivation almost similar to those of the control I_{Na}. The voltage dependence of the slow I_{Na} is 40 mV more negative than that of the fast one. These results suggest that PoTX affects all the sodium channels and that the fast and slow I_{Na}-components originate from a possible PoTX-induced interconversion between a fast and a slow operating mode of the sodium channel (*40*).

Discussion

The present paper describes two types of arthropod neurotoxins selected by nature to incapacitate insects and spiders. The first group consists of low molecular weight neurotoxins that contain a polyamine part. These toxins are highly specific for modifying the kinetics of cation channels coupled to excitatory receptors like glutamate or acetylcholine receptors. Their action is

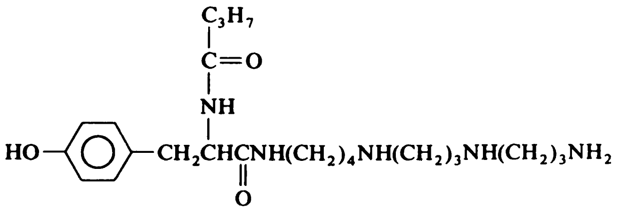
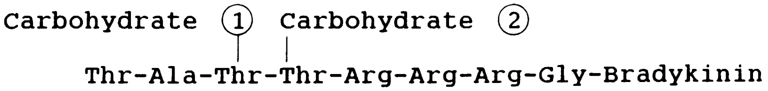


Figure 1. Structure of δ-philanthotoxin (philanthotoxin-4.3.3) isolated from the wasp *Philanthus triangulum.*



Carbohydrate ① Carbohydrate ②

Thr-Ala-Thr-Thr-Arg-Arg-Arg-Gly-Bradykinin

Carbohydrate 1 : N.Ac.Galactosamine 1-2, Galactose 1

Carbohydrate 2 : N.Ac.Galactosamine 2-3, Galactose 2

Bradykinin is Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Figure 2 . Structure of vespulakinin 1 (*33*).

A Carbohydrate free peptide (VSK 1)

B (Galα)Thr^3 - VSK 1

C (Galα)Thr^3, (Galα)Thr^4 - VSK 1

D (Galβ)Thr^3 - VSK 1

Figure 3- Synthetic vespulakinin-1-analogues (*34, 35*).

Phe—Leu—Pro—Leu—Leu
|
Leu—Ser—Gly—Leu—Ile
|
Leu—Met—Thr—Pro—Pro
|
Ile—Ala—Gln—Ile—Val
|
His—Asp—Ala—Gln—Arg
|
NH_2

Figure 4- Amino acid sequence of poneratoxin (PoTX), a neurotoxin isolated from the ant *Paraponera clavata.*

non-competitive and reversible. Attempts to change the molecules into more potent antagonists resulted in an increase in potency with factors varying from only 10 to 100 (*8, 10-12*). If no further increase in potency and/or decrease in the reversibility can be obtained, these interesting class of neurotoxins may be only important as tools for the study of insect neurophysiology.

The second group of medium-sized low molecular weight arthropod neurotoxins consists of polypeptides. Examples of such neurotoxins are the (wasp) kinins (*27-29*) and the ant venom component poneratoxin (*38-40*). It is conceivable that a large number of potent polypeptide neurotoxins could be isolated from venoms of insectivore ants. Especially in case of irreversible or slowly reversible action of primary synthesis products, as has been demonstrated for kinins, a pesticide action could be obtained by means of genetic manipulation. Moreover, it might be possible to design a mimic pharmacophore (loop) of a peptide (*44*).

Literature Cited

1. Van Marle,J.;Piek,T.;Lind,A.;Van Weeren-Kramer,J.*J.Insect Physiol.***1982**, *28*,343-347.
2. VanMarle,J.;Piek,T.;Lind,A.;VanWeeren-Kramer,J.*Comp.Biochem.Physiol.* **1983**,*74C*, 191-194.
3. Van Marle,J.;Piek,T.;Veldsema-Currie,R.D.*Exp.Brain Res.***1986**,*62*,25-28.
4. Piek,T.;Fokkens,R.H.;Karst,H.;Kruk,C.;Lind,A.;Van Marle,J.;Nakajima,T.; Nibbering, N.M.M.;Shinozaki,H.;Spanjer,W.;Young,Y.C. In *Neurotox'88: Molecular Basis of Drug & Pesticide Action*;Editor G.G.Lunt.Excerpta Medica Int.Congress Series 832,Elsevier Amsterdam 1988.
5. Clark,R.B.;Donaldson,P.L.;Gration,K.A.F.;Lambert,J.J.;Piek,T.;Ramsey, R.;Spanjer,W.; Usherwood,P.N.R.*Brain Research* **1982**, *241*,105-114.
6. Piek,T.*Comp.Biochem.Physiol.***1982**,*72C*,311-315.
7. Eldefrawi,A.T.;Eldefrawi,M.E.;Konno,K.;Mansour,N.A.;Nakanishi,K.;Oltz,E.; Usherwood,P.N.R.*Proc.Natnl.Acad.Sci.*USA **1988**,*85*,4910-4913.
8. Bruce,M.;Eldefrawi,A.T.;Eldefrawi,M.E.;Bukownik,R.;Goodnow,R.;Nakanishi, K.; Usherwood, P.N.R.*Toxicon* **1990**,*28*,1333-1346.
9. Anis,N.;Sherby,S.;Goodnow,R.;Niwa,M.,Konno,K.;Kallimopoulos,T.; Bukownik,R.;Nakanishi,K.;Usherwood,P.N.R.;Eldefrawi,A.;Eldefrawi,M.J. *Pharmacol.Exp.Therapeut.***1990**,*254*,764-773.
10. Karst,H.;Piek,T.;Van Marle,J.;Lind,A.;Van Weeren-Kramer,J.*Comp.Biochem. Physiol.***1991**,*98C*,471-477.
11. Karst,H.;Piek,T.;*Comp.Biochem.Physiol.***1991**,*98C*,479-489.
12. Nakanishi,K.;Goodnow,R.;Konno,K.;Niwa,M.;Bukownik,R.;Kallimopoulos, T.A.; Usherwood,P.N.R.;Eldefrawi,A.T.;Eldefrawi,M.E.*Pure Appl.Chem.* **1990**, *62*,1223-1230.
13. Benson,J.A.;Schürmann,F.;Kaufmann,L.,Gsell,L.;Piek,T.*Comp.Biochem. Physiol.*1992,in press.
14. Karst,H.;Piek,T.;Van Marle,J.*Neurosc.Res.Comm.***1990**,*7*,69-74.
15. Volkova,T.M.;Grishin,E.V.;Arseniev,A.S.;Reshetova,O.S.;Onoprienko,V.V. *Proc.6th Europ.Soc.Neurochem.Meet.***1986**,p.189.
16. Grishin,E.V.;Volkova,T.M.;Arseniev,A.S.*Toxicon* **1989**,*27*,541-549.
17. Skinner,W.S.;Dennis,P.A.;Lui,A.;Carney,R.L.;Quistad,G.B.*Toxicon* **1989**,*28*,541-546.

18. Ragsdale,D.;Gant,D.B.;Anis,N.A.;Eldefrawi,A.T.;Eldefrawi,M.E.;Konno,K.; Miledi,R. *J.Pharmacol.Exp.Therapeut.* **1989**,*251*,156-163.
19. Schluter,N.C.M.;Van Weeren-Kramer,J.;Piek,T.;Van Marle,J.*Comp.Biochem. Physiol.* **1992**,*101C*,35-39.
20. Schluter,N.C.M.;Piek,T.;Lopes da Silva,F.H.*Comp.Biochem.Physiol.* **1992**, *101C*,41-47.
21. Van Wilgenburg,H.;Piek,T.;Mantel,P.*Comp.Biochem.Physiol.* **1984**,*79C*,205-211.
22. Piek,T.;Hue,B.*Comp.Biochem.Physiol.* **1989**,*93C*,403-406.
23. Rozenthal,R.;Scoble,G.T.;Albuquerque,E.X.;Idriss,M.;Sherby,S.;Sattelle,D.B.; Nakanishi,K.;Konno,K.;Eldefrawi,A.T.;Eldefrawi,M.E.*J.Pharm.Exp. Therapeut.* **1989**,*249*,123-130.
24. Jaques,R.;Schachter,M.*Brit.J.Pharmacol.* **1954**,*9*,53-57.
25. Schachter,M.;Thain,E.M.*Brit.J.Pharmacol.* **1954**,*9*,352-359.
26. Udenfriend,S.;Nakajima,T.;Pisano,J.J.*Proc.Int.Congr.Biochem.*7th VIII-4,**1967**,501.
27. Nakajima,T.In *Venoms of the Hymenoptera,Biochemical,Pharmacological and Behavioural Aspects*;Editor T.Piek;Academic Press,London Chapt.6 pp.309-327.
28. Piek,T.;Hue,B.;Mony,L.;Nakajima,T.;Pelhate,M.;Yasuhara,T.*Comp.Biochem. Physiol.* **1987**, *87C*,287-295.
29. Yasuhara,T.;Mantel,P.;Nakajima,T.;Piek,T.*Toxicon* **1987**,*25*,527-535.
30. Piek,T.;Hue,B.;Mantel,P.;Nakajima,T.;Pelhate,M.;Yasuhara,T.*Comp. Biochem. Physiol.* **1990**,*96C*,157-162.
31. Piek,T.;Schmidt,J.O.;De Jong,J.M.;Mantel,P.*Comp.Biochem.Physiol.* **1989**, *92C*,117-124.
32. Hue,B.;Piek,T.*Comp.Biochem.Physiol.* **1989**,*93C*,87-89.
33. Yoshida,H.;Geller,R.G.;Pisano,J.J.*Biochemistry* **1976**,*15*,61-64.
34. Rocchi,R.;Biondi,L.;Filira,F.;Scolaro,B.*Int.J.Pept.Prot.Res.* **1987**,*30*,240-256.
35. Biondi,L.;Cavaggion,F.;Filira,F.;Rocchi,R.*Gazz.Chim.Ital.* **1988**,*118*,427-433.
36. Gobbo,M.;Biondi,L.;Filira,F.;Scolaro,B.;Rocchi;Piek,T.*Int.J.Pept.Prot.Res.* **1992**,in press.
37. Schmidt,J.O.In *Venoms of the Hymenoptera Biochemical,Pharmacological and Behavioural Aspects*;Editor T.Piek;Academic Press London,Chapt.6 pp.425-508.
38. Piek,T.;Hue,B.;Mantel,P.;Nakajima,T.;Schmidt,J.O.*Comp.Biochem.Physiol.* **1991**,*99C*, 481-486.
39. Piek,T.;Duval,A.;Hue,B.;Karst,H.;Lapied,B.;Mantel,P.;Nakajima,T.; Pelhate,M.; Schmidt,J.O.*Comp.Biochem.Physiol.* **1991**,*99C*,487-495.
40. Duval,A.;Malécot,C.O.;Pelhate,M.;Piek,T.*Pflügers Arch.* **1992**,in press
41. Piek,T.In *Neuropharmacology of Insects*; Publisher:Pitman,London,**1982**.Ciba Found.Symp.88 pp.275-290.
42. Piek,T.*Pestic.Sci.* **1987**,*19*,317-322.
43. Hildebrand,J.G. In *Neurotox'88:Molecular Basis Drug & Pesticide Action*; Editor G.G.Lunt;Elsevier,Amsterdam Exerpta Medica Int.Congr.Ser 832 pp.583-588.
44. Milner-White,E.J.*TIPS* **1989**,*10*,70-74.

RECEIVED October 6, 1992

Chapter 17

Viral Insecticides

James L. Vaughn

Insect Biocontrol Laboratory, Plant Sciences Insitute, Beltsville Agricultural Research Center–West, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705–2350

Viruses are potentially valuable tools for managing pest insect populations. As has been observed in countless natural epizootics, a virus can cause significant reductions in pest insect populations. Presently they are used on a large scale to control the pine caterpillar in Japan and the soybean looper in Brazil. Four are registered for use in the U.S. They are exempted from the requirement for residue tolerance and, therefore, are free of residue problems. Their host specificity makes them compatible with other natural enemies and safe to use in environmentally sensitive areas. Recent advances in genetic manipulation offer possible solutions to the problems of severely restrictive host range and slow activity and have increased the attractiveness of viruses as pest control agents. Although resistance is possible, the fact that the virus is capable of variation and change reduces this possibility. The technology for *in vitro* production has improved rapidly and appears to be economically feasible.

The control of insect pests with viruses has a long history. In 1892 there was a severe outbreak of the nun moth, *Lymantria monacha*, in the pine forests of Germany. A disease of this insect, believed to be caused by an organism called Hofmann's bacterium, had been observed. To control the pest, foresters attempted to cultivate the suspected causative organism on a mixture of horse meat and potatoes. Following an appropriate incubation, pieces of the meat were tied in the trees in threatened parts of the forest. After a time it was reported that larvae of the nun moth began to show

This chapter not subject to U.S. copyright
Published 1993 American Chemical Society

typical signs of the disease. Eventually the population of nun moths collapsedand extensive damage to the forest was prevented. So impressive was this result that the technique was widely used by the German forest service for nun moth control, with highly variable results (*1*). We now know that the disease was caused by a virus and that the collapse of the population was due to naturally occurring virus not to the intervention of the foresters. The story does illustrate two important points, however. First, attempts to use viruses to control insect pests often have been made with little or at least incomplete understanding of the biology of the system being used. Second, when this was done, the results were often inconsistent and failures occurred frequently.

The long standing interest in the use of viruses for controlling insect pests is not surprising. Many viruses cause acute diseases and the affect they can have in a dense pest population often is spectacular. In Ontario, Canada a virus of the European spruce sawfly, *Gilpinia herecynia*, that was introduced from Europe in 1950 causes annual epizootics and still maintains the insect population below a level that would cause economic damage (*2*). A similar virus periodically causes the collapse of gypsy moth, *Lymantria dispar*, populations in hardwood forests (*3*). A virus causes regular, naturally occurring epizootics in populations of the cabbage looper, *Trichoplusia ni* (*4*). Over 1600 virus isolates have been recorded from diseased animals in about 1100 species of insects and mites (*5*).

There are four characteristics that a virus must have to make it useful as a pest management tool. First, it should be lethal to the target insect or stop it from feeding. Second, the incubation period should be short. A prolonged incubation period in which the insect continues to feed may result in economic loss, even though the pest may be eventually controlled. Third, the virus must be safe to use. It must not infect vertebrates, plants, and nontarget insects. Finally, it must be stable enough to retain activity for a reasonable time between production and application.

The viruses that cause diseases in insects are presently classified into several families shown in Table I. The Poxviridae, Reoviridae, and Baculoviridae have received most of the attention as biological pest control agents. One feature that these viruses have in common is that the virion is occluded in a protein crystal when the replication is completed giving a relative stability to the virion, Figure 1.

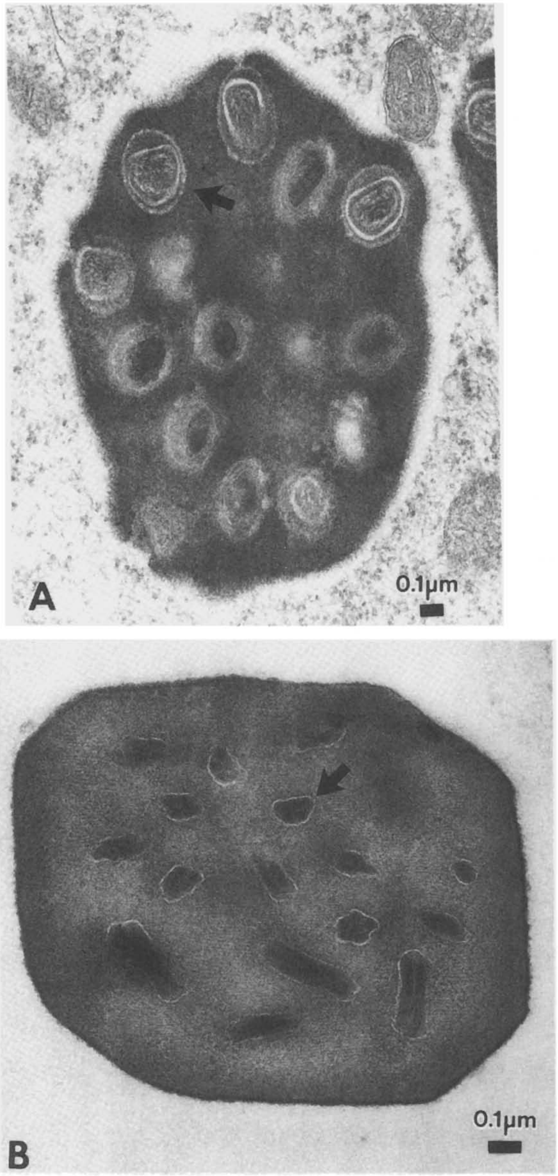
Insect viruses belonging in the family Poxviridae have morphological and enzymatic characteristics very similar to other members of this family that have been isolated from vertebrates. However, the restriction enzyme nucleotide fragment patterns are quite different and the Entomopox viruses have not been shown to infect any animals other than insects (*6*). Pox viruses have been described in a number of pest insects including forest pests, beetles, and grasshoppers (as examples see refs. *7-9*). These viruses develop slowly in the infected insect. For example, Henry, et al. (*9*) reported that death of infected grasshoppers occurred 20-30 days after inoculation. The similarity of these viruses to vaccinia, the long incubation time, and problems inproduction have discouraged their development for pest control.

Table I. Viruses Infecting Insects

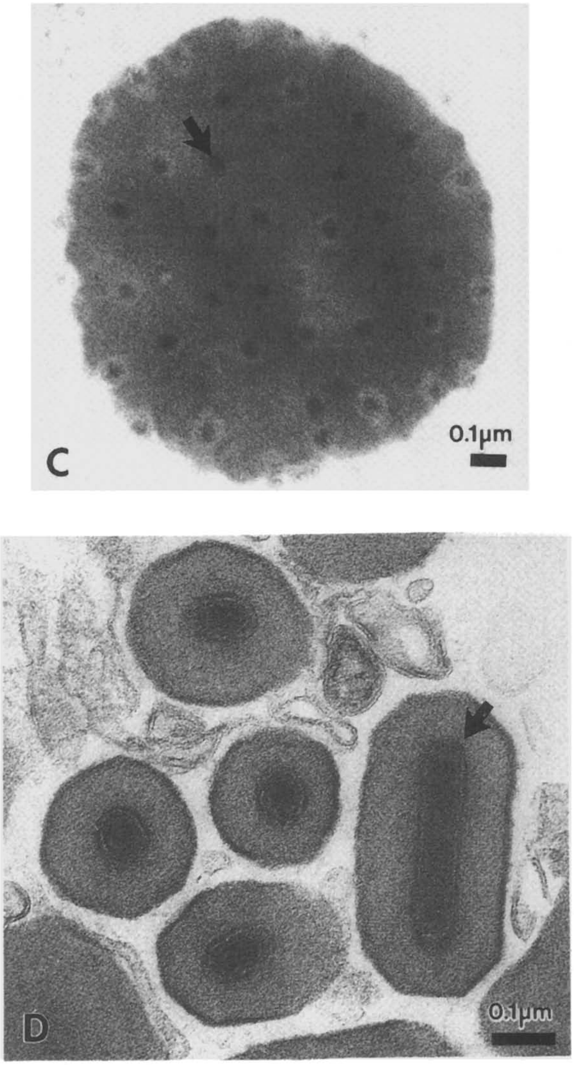
FAMILY	NUCLEIC ACID	INSECT PATHOGENS
BACULOVIRIDAE	*ds*DNA[a]	Nuclear Polyhedrosis, Granulosis virus, non-occluded
BIRNAVIRIDAE	*ds*RNA	
IRIDOVIRIDAE	*ds*DNA	Iridovirus, Chloriridovirus
NODAVIRIDAE	*ss*RNA	Nodaviridae virus
PARVOVIRIDAE	*ss*DNA	Densovirus
PICORNAVIRIDAE	*ss*RNA	
POLYDNAVIRIDAE	*ds*DNA	
POXVIRIDAE	*ds*DNA	Entomopox
REOVIRIDAE	*ds*RNA	Cytoplasmic Polyhedrosis

[a] ds = double stranded; ss = single stranded

Viruses in the family Reoviridae are especially interesting in that they cause diseases in plants, vertebrates, and invertebrates (*10*). A unique characteristic of the viruses in this family that multiply in insects is the virions are occluded in a protein crystal, polyhedra, formed in the cytoplasm prior to release from the infected cell. For this reason they are called cytoplasmic polyhedrosis viruses (CPV). About 200 species of insects have been found to be infected by a CPV. Eighty-five percent of these are Lepidoptera; the remainder infect Diptera, Hymenoptera, and Coleoptera (*11*). Only a few of these have been suggested for use as biocontrol agents and only one, the CPV of the pine caterpillar, *Dendrolimus spectabilis*, has been registered in Japan



Figures 1A and 1B. Electron micrographs showing the inclusions of the infectious virions (arrows) in the protein crystal. A) Entomopox virus from the army cutworm, *Euxoa auxiliaris.* B) Nuclear polyhedrosis virus from the alfalfa looper, *Autographa californica.* All photographs are courtesy of Dr. Jean R. Adams, Insect Biocontrol Laboratory. Figure 1B is reprinted from the *Atlas of Invertebrate Viruses (60),* with permission of CRC Press.



Figures 1C and 1D. C) Cytoplasmic polyhedrosis virus from the gypsy moth, *Lymantria dispar.* D) Granulosis virus from the codling moth, *Cydia pomonella.* All photographs are courtesy of Dr. Jean R. Adams, Insect Biocontrol Laboratory.

as a microbial insecticide. Generally these viruses are chronic and persist at low levels in host populations. Diseased larvae are not as active and feed less than uninfected larvae. The larvae may survive and pupate, but the adults from these pupae are often malformed and lay fewer eggs than uninfected adults. Thus, control may require replication in several generations of the pest, and the use of the virus should be on crops where some insect damage can be tolerated so that these characteristics can be exploited.

The only family of viruses found exclusively in arthropods is the Baculoviridae. These viruses have been isolated most frequently from insects, and rarely from shrimp (*12*). Baculoviruses usually cause acute infections, and larvae infected in the first or second instar seldom survive to adults. These viruses also have the other desirable characteristics required for a potential control agent; the infectious virion is protected with the protein crystal and the host range is restricted to Arthropods. Baculoviruses infecting most of the serious crop pest species have been identified. The genetics of the baculoviruses are the best known of the insect viruses, and methods for the alteration of the genome of several baculoviruses with recombinant DNA technology are available. At the present time these viruses are the most likely candidates for use in pest control and the rest of this discussion will deal with this group.

Characterization of Baculoviruses

As the family name implies, these are rod shaped viruses. The nucleic acid is a single molecule of circular, double stranded DNA with a molecular weight of 90-230 kb contained in nucleocapsids 250-300 nm in length and 30-60 nm in diameter. The virion consists of one or more nucleocapsids enclosed in a single envelope. Enveloped virions are enclosed in a crystalline protein viral occlusion which usually is polyhedral in shape. The crystal consists of a single protein with a molecular weight of 25-33 x 10^3.

The Family is subdivided based upon morphological characteristics. Those viruses that have one nucleocapsid per virion and have only one or rarely two virions per inclusion body are in the genus Granulosis Virus (GV). The inclusion bodies of these viruses are ovicylindrical. They have been isolated from a number of pest insect species including *Pieris brassicae* and *Cydia pomonella*. These viruses are highly host specific, infecting only one or a few closely related species. They have been isolated from about 50 species of Lepidoptera.

Viruses with more than one virion per inclusion body are placed in the genus Nuclear Polyhedrosis Viruses (NPV). This genus is further divided between those viruses with a single nucleocapsid per envelope (SNPV) and those with multiple nucleocapsids per envelope (MNPV). The SNPV seem to have a somewhat broader host range than the GV, but are still generally restricted to closely related species. They have been isolated from a number of species in seven insect orders including such important pest species as

Heliothis virescens and *Helicoverpa zea*. In addition isolations have been made from Crustacea (*12*).

The MNPV are the most widely distributed and the best known. There have been isolations made from 400-500 insect species including such serious insect pests as *Spodoptera frugiperda*, *Helicoverpa armigera*, *Lymantria dispar*, and *Mamestra brassicae*. The host range varies from a single known species as the *L. dispar* NPV to the *Autographa californica* NPV that will infect 43 insect species and many insect cell lines (*13*, *14*).

Advantages to Using Viruses in Pest Management

Host-Specificity. The infectivity of baculoviruses for insects other than the insect species from which they were isolated has been widely tested. Although not all data for any specific virus are acceptable because of insufficient test conditions, some conclusions can be made. This subject was reviewed in depth by Groner (*15*) and only will be summarized here. With only a few exceptions, infectivity of most baculoviruses were restricted to the genus or family of the original host. An early review of Ignoffo (*16*), cited by Groner, reported that only in three of nine attempts were baculoviruses transmitted to insects in orders other than the original, and 30 of 137 attempts to transmit to new families were successful.

The *H. zea* NPV was extensively tested against mammals and birds as part of the requirements for registration by the Environmental Protection Agency (EPA). These tests included acute and subacute toxicity and pathogenicity tests in mammals, teratogenicity and carcinogenicity tests, and tests against a variety of wildlife and aquatic invertebrates. Similar tests have been carried out with other viruses. There has been no indication from any of this testing that the baculoviruses can cause disease in animals other than insects.

A baculovirus is much less likely to reduce the control provided by existing insect parasites and predators than synthetic insecticides. Thus, it will not increase the need for control measures for either the target pest or other potential pests in the same crop. It poses no hazard to fish, birds or other wild life, and it will not accumulate in organisms higher up the food chain as some chemicals do. It is not a threat to humans during production or application if procedures to prevent the development of adventitious microorganisms and to contain allergens are followed.

No Residue Problems. Baculoviruses, like other microbial control agents, have the potential to multiply after application. They have also been shown to occur on harvested crops as the result of naturally occurring disease in the pest population (*4*). Therefore, when these organisms are to be used to control pests, EPA requires that they meet the requirements for an exemption from the requirement of tolerance. As a result, microbials can be used up to and including the day of harvest.

Successful Use of Baculoviruses

Of the several hundred baculoviruses causing diseases in insects, only a few have been used successfully against pests. Yearian and Young (*17*) reviewed the reports on those viruses with demonstrated control potential and found that of 31 viruses identified 29 were baculoviruses. With one exception all the pests were Lepidoptera and one half of these were in the family Noctuidae. Of those viruses used in agriculture, 10 were on field crops, 10 on vegetable crops, 7 on forage crops, and 6 on fruit crops. Entwistle and Evans (*18*) have assembled a list of 39 pests that could be controlled with baculoviruses (Table II.) This list contained 32 Lepidoptera, 6 Hymenoptera, and one Coleoptera. Of the 39, 16 are forestry pests and the remaining 23 are agriculture pests. Selected examples from this list are discussed in some detail to illustrate the level of control obtained with viruses.

Cole Crops. The *Trichoplusia ni* MNPV and the *A. californica* MNPV have consistently been shown to suppress cabbage looper populations on cole crops. In studies by McEwen and Hervey (*19*) populations of *T. ni* on cabbage, broccoli, and cauliflower were reduced by 75-98% with *T. ni* NPV. Vail and his coworkers (*20*) reported that *A. californica* NPV or *T. ni* NPV used alone or in combination reduced looper populations on cabbage as well as the standard chemical insecticides. However, the initial control was not as good and perhaps, as a result, damage control was not as good as with chemical insecticides. Using a 10-fold higher treatment, Hostetter et al. (*21*) obtained 93% marketable cabbage heads compared to only 30% in the control plots.

A second pest frequently found on cole crops worldwide is the imported cabbageworm, *Pieris rapae*. This insect is not affected by the NPVs but is killed by a granulosis virus, another baculovirus. On cabbage, control of the cabbageworm with two applications of this virus was equal to that with DDT followed by organophosphate insecticide (*22*). Treatment with the virus reduced the number of insects by 87-97% nine days post-treatment. The damage was confined to the wrapper leaves and the marketable heads were increased from 22% to 84%. Note that the data on population reduction was given for nine days after treatment. Although feeding stops, the insects may live for some time after exposure to the virus. This must be understood by the user.

The diamondback moth, *Plutella xylostella*, a serious pest of cole crops in much of the world, is rapidly developing resistance to chemical insecticides and to the endotoxin of *Bacillus thuringiensis* (*23*). This insect has been shown to be susceptible to both the *A. californica* NPV (*24*) and to a GV (*25*). Recently in India studies demonstrated that an unidentified, locally isolated NPV was highly infectious for diamondback larvae in laboratory tests (*26*). Since resistance is rare to viruses, this may be the way to control this difficult pest.

Table II. Agricultural Pests Potentially Controllable with Baculoviruses

PEST	PLANT
NPV Baculovirus	
Anticarsia gemmatalis	soybean
Choristoneura fumiferana	spruces
Colias philodice eurytheme	alfalfa
Galleria mellonella	beehives
Gilpinia hercyniae	spruces
Helicoverpa armigera	cotton, sorghum
Helicoverpa virescens	cotton
Helicoverpa zea	cotton, vegetables
Heliothis punctigera	cotton
Hyphantria cunea	broadleaved
Kotochalia junodi	black wattle
Lymantria dispar	broadleaved
Lymantria fumida	Japanese fir
Malacosoma distria	aspen
Malacosoma fragile	orchards
Mamestra brassicae	brassicas
Neodiprion lecontei	pines
Neodiprion sertifer	pines
Neodiprion swainei	Jack pine
Neodiprion taedae linearis	lobolly pine
Orgyia pseudotsugata	Douglas fir
Spodoptera exigua	horticultural crops
Spodoptera littoralis	cotton
Spodoptera litura	bananas
Thymelicus lineola	timothy grass
Trichoplusa ni	brassicas
Wiseana cervinata	pasture
GV Baculovirus	
Agrotis segetum	carrots, beets
Choristoneura muriana	spruces
Cydia pomonella	apples, pears, walnuts
Phthorimaea opercullela	potatoes
Pieris rapae	brassicas
Pieris brassicae	brassicas
Plutella xylostella	brassicas
Plodia interpunctella	stored products
BV Baculovirus	
Oryctes rhinoceros	coconuts

Per Entwistel and Evans (18)

American Chemical
Society Library
1155 16th St. N. W.
Washington, D.C. 20036

Orchard Crops. The most promising virus control of an orchard pest is the GV of the apple codling moth, *C. pomonella*. The insect is a pest of apples, pears, and walnuts all over the world. In many places it's natural enemies are incapable of keeping populations below economic tolerance (*1*). The virus is highly specific and one of the most virulent known. The first field trials were conducted by Falcon and his associates in California (*27*) with virus isolated from larvae collected in Mexico (*28*). It has subsequently been tested in Australia, Canada, South America, and Europe. Over a five year period, the GV outperformed azinphosmethyl in a commercial pear orchard (*29*). The potential of this virus has been thoroughly demonstrated and it would appear to be a strong candidate for development.

Cotton. In the early 1960's the work of Ignoffo and colleagues provided the stimulus for the development of the *H. zea* NPV for the control of *H. zea* and *H. virescens* on cotton (*30*). Early field results were erratic but promising. In 1969, excellent results were reported in large-scale field tests (*31*). Application of the virus at 247 LE/acre in 1971 resulted in much more square and boll damage than 123.5 and 247 LE/acre applied in 1972 (*32*). By 1974 improved formulations had been developed, and in field tests at several locations seed cotton yields were significantly higher than with previous formulations. In 1975 test yields were consistently better except where the insect populations were high (Reviewed by Yearian and Young, *17*). The *H. zea* NPV became the first virus to be registered for use in the United States to control insect pests in 1975 and was the only virus manufactured and sold commercially.

Bell and Romine (*33*) have tested combinations of the NPV and the exotoxin of *B. thuringiensis* and reported that there was no apparent benefit to the use of combinations of pathogens to control *Heliothis* in cotton. All treatments reduced the number of larvae when compared to the untreated control. However, the virus alone did not give significant reduction of terminal damage. When they used the virus - bacterial mixture, applied by air, to treat a commercial field of short-staple cotton the number of eggs, the damage to bolls and the yields from the field were about the same as in the fields treated with chemical insecticides.

In Egypt, *Spodoptera littoralis*, the Egyptian cotton leafworm, is a serious pest on cotton and a NPV has been developed to provide an alternative control measure. Applications of 5×10^{12} polyhedra/ha to the under surface of the leaves were as effective as chemicals in preventing damage. The virus did not remove the insect predator population as did the chemicals and as a result large numbers of beneficial insects were found in neighboring fields and reduced the number of egg masses below the level required to cause significant damage without additional treatment (*34*).

Soybeans. The velvetbean caterpillar (*Anticarsia gemmatalis*) defoliates over 11 million ha of soybean per year in Brazil and most of the chemical insecticides applied to soybeans are for its control. In 1979 a program was

In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.

established at the Soybean National Research Center of Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA) to develop alternative non-polluting methods for its control. An NPV, isolated from veletbean caterpillars in Brazil, was evaluated for efficacy and safety at the Center. A program was begun to produce the virus at the Center for distribution to extension service personnel and farmer cooperatives for further multiplication and field application.

The project began with the treatment of 2,000 ha in the 1982/83 season. By the 1987/88 season 500,000 ha were treated. Due to increasing farmer demand the production programs had to be expanded and the technology was transferred to six private firms. The process also has been transferred to other South American countries, such as Argentina and Paraguay, where 2,000 and 18,000 ha, respectively are treated with the virus (*35*). A commercial formulation of the NPV has been manufactured and sold in Brazil. In 1988/89 the amount sold was sufficient to treat about 81,000 ha.

Forests. The forest environment is ideal for biological control because minor cosmetic damage and the presence of low numbers of insects can be tolerated. Since the attempts by the German foresters to control the nun moth with virus 100 years ago, viruses of numerous other pests have been used in pest management systems. The classical introduction into the U. S. and Canada of the NPV of the European spruce sawfly has already been mentioned. An NPV for use against the Douglas fir tussock moth, *Orgyia pseudotsugata*, and a second NPV for use in controlling European pine sawfly, *Neodiprion sertifer*, have been registered by EPA. Another virus for controlling the redheaded pine sawfly, *N. lecontei*, has been developed in Canada.

In the 1860's the gypsy moth, *Lymantria dispar*, was introduced into the United States and became a serious defoliator of Northeastern forests, parks, and urban areas. In the latter two areas, it is also an aesthetic problem when present in high numbers. It was observed that the NPV was one of the causes of the periodic collapse of high density populations and in the 1960's research began to develop this virus for managing gypsy moth. This research culminated in the U. S. Forest Service obtaining registration of the virus as a pesticide. However, a combination of low virulence and very limited availability restricted the use. Recently a new strain of this NPV was obtained by selection from a wild population of the virus that has increased virulence in laboratory tests and may provide control at application rates that are economical (*36*). The addition of a stilbene to the tank mixture before application increases the activity of the virus and reduces the dose needed to control gypsy moth further enhancing this virus as a successful control agent (*37*).

Restraints to Development

The viruses discussed above are selected examples of recent progress in the use of viruses for pest management. Despite the economic importance of the crops affected by the pests listed and the potential value of viruses for their control, only five of those in Table 2 have been registered and approved for use in North America. The five are the NPVs for *H. zea*, *L. dispar*, *O. pseudotsugata*, *N. lecontei*, and *N. sertifer*. In 1985 the worldwide sale of viruses for pest control was estimated at about $1 million US compared to the sales of products containing the bacterium *B. thuringiensis* which were estimated at $30 million US of a total insecticide sales of $5 billion US (*38*). None are currently sold for general use in the United States.

A market report by Frost and Sullivan (New York) cited by Reinecke (*39*) forecasts that by the year 2000 biologicals will be 40% of the pesticide sales assuming that the total market remains unchanged for the next ten years. Insect resistance to chemicals, public concern about synthetic chemical residues on food, and environmental pollution have come together to establish a need for biologicals. Successful marketing requires the appropriate product to meet a need. Years of basic research, briefly described here, have developed the raw materials, viruses that kill insects. The viruses can capture a significant share of the increased biological insecticide sales if the factors constraining their use are overcome.

Specificity. The specificity which makes the viruses attractive as an environmentally safe insecticide is also a limitation to commercialization. In many situations the viral products will be minor use pesticides and the potential market size will not justify the cost of development and registration. For these viruses it may be imperative that government or institutional funds support some or most of the development. The U. S. Forest Service supported the development of and holds the registrations for three of the four viruses registered in the United States. In California a unique partnership has supported the development of the GV of the codling moth. California farmers developed a non-profit cooperative to provide funds for the development of the virus. The experimental use permit was issued to the University of California, Berkeley and UCB is the potential registrant (*29*). Increased use of funds from the federal IR-4, minor use pesticide program for the collection of data on biologicals will also further the development of viral pesticides.

From the end user's view, a product that only controls one pest on a crop with several major insect pests is not as desirable as one that controls them all. This can be overcome by using a combination of specific pesticides. However, growers need to be made aware that switching to a broad spectrum pesticide in this situation may remove the natural enemies of the pests and also increase their pest control costs in the long term.

Slow Action. Unlike synthetic chemicals or the *B. thuringiensis* toxin, viruses are pathogens and the target insects are killed as the end result of a disease. Growers are accustomed to the rapid knock-down and death caused by the chemical pesticides and need to be educated on the effects to be expected when pathogens are used. Viruses applied to rescue a crop already suffering damage from pests will not give satisfactory results even if there is significant reduction in pest numbers because the insects will feed for two or more days during the incubation period of the disease. Similarly, insects that feed on foliage for only a few days before boring into fruit or other parts of the plant may cause economic loss during the time between exposure to the virus and death. Thus, the timing of the application is much more critical for viruses than for chemicals.

If there is sufficient understanding of the biology and ecology of the pest and an economic threshold has been established on the threatened crop, economic loss can be minimized by proper timing of the application. The monitoring of pest numbers, knowledge of the rate of increase in pest numbers, and the length of the incubation period are needed to time the application of viruses. Viruses are most infectious for neonatal or early instar larvae. Therefore, the virus should be applied as soon after hatching as the need can be established. This also will reduce the loss caused by foliage feeding insects, as they do the most damage in the later instars.

There has been great interest in using recombinant DNA technology to modify the baculoviruses to prevent loss during the incubation period. With this technology, a foreign gene coding for a protein that causes the insect to stop its activity shortly after infection occurs could be inserted into a nonessential area on the viral genome. The gene should be expressed very early in the replication cycle but allow continued development of the virus.

Several types of proteins, affecting insects in different ways are possible candidates: insect growth regulating proteins, regulators of essential physiological functions, and those that are toxic to the target insects. An example of the first type of protein is the juvenile hormone esterase. This enzyme is produced in the last larval instar and hydrolyzes the active juvenile hormone to the inactive acid form. As a result of the reduced levels of the hormone, the larva stops feeding and metamorphosis is initiated. Hammock et al. (*40*) constructed a modified AcNPV that produced the enzyme during replication. When first instar *T. ni* larvae were fed this virus the feeding and growth of the infected larvae were halted in the third instar. These larvae were much smaller than larvae killed by the wild type virus.

The diuretic hormone used by Maeda (*41*) is an example of the second type of proteins. This hormone controls the intake and excretion of water. When insects were fed a recombinant virus containing the gene for the hormone all larvae died four days after inoculation, whereas 95% of the control larvae were still alive. Decreased volumes of collectable hemolymph, fragile midguts and other physiological changes indicated that a disruption of the process controlling water balance had occurred.

The insertion of genes for insect specific neurotoxins has been an intriguing concept for the improvement of the baculoviruses as control agents. Several reports of initial successes in modifying the *A. californica* NPV in this manner were published recently. Tomalski and Miller (*42*) inserted the gene for mite neurotoxin in place of the viral gene for polyhedrin in the *Ac*NPV. The gene was expressed early after infection, caused paralysis, and eventually the larvae died of the viral disease. This viral construction did not produce polyhedrin and so no occluded virions were produced. The non-occluded virion is not infectious to the insect per os, but must injected and therefore is not suitable for insect control. A second polyhedrin positive recombinant was produced in which both the neurotoxin and polyhedrin genes were expressed. Neonatal larvae were infected per os with this virus and showed paralysis by the second day post infection.

A similar construction was made with the NPV of the silkworm, *Bombyx mori* by Maeda and associates (*43*) in which the gene for an insect specific toxin from the scorpion, *Androctonus australis* was inserted into the viral genome in the site of the polyhedrin gene. When silkworm larvae were injected with the recombinant virus the expressed toxin caused feeding cessation within 40hr post infection followed by paralysis and death by 60 hr. Larvae infected with control virus did not die for another 36 hr.

Stewart and colleagues (*44*) have also constructed a recombinant in which they inserted the gene for this scorpion peptide. Their constructs were polyhedrin positive. Per os infected insects became paralysed and the LT_{50} was reduced from 113 hr for wild type virus to 86 hr for the recombinant. In experiments in which infected larvae were placed on cabbage leaves after feeding overnight on a diet plug inoculated with 10^4 polyhedra, those larvae fed recombinant virus caused 50% less damage than those infected with the wild virus. Studies on another set of constructs containing this gene demonstrated that the expressed toxin was effective in reducing the LT_{50} of the *Ac*NPV in *H. virescens* from 125 hr for wild type virus to 88 hr for the recombinant (*45*).

These studies are the first to demonstrate increased effectiveness with recombinant viruses. If the increase demonstrated in these laboratory studies carries on in field tests, this technique has potential for increasing the use of the NPV in the insecticide type strategy of pest management. As a result, commercial concerns have increasing interest in developing products based on these viruses.

Production. Poor supply and high cost are factors that have limited the development of viruses in pest management. Viruses are obligate parasites, as such, their multiplication occurs only in living cells, either in the insect or in cultures of insect cells. Producing viruses in mass reared insects is generally labor intensive. There has been more interest in the insect system in developing countries where inexpensive labor is available and chemical insecticides are more expensive. The method is suitable for a cottage industry type production unit and the very successful program to control the velvetbean caterpillar in Brazil began with the production of the virus in the farmer cooperatives using seed virus provided by the research laboratory in EMBRAPA (*35*). Similar programs have been used in Thailand, Columbia, Zimbabwe, and China (*46*). In China the total area treated with viruses is now near 70,000 ha. A manufacturing facility has been established to produce the *H. armigera* NPV (Gu Baogen, personal communication).

In countries outside of North America the commercial production of viruses has an increasing role in pest management as the development of the NPV for control of the velvetbean caterpillar demonstrates. In Guatemala, two viral products are produced and sold by a private company. One based on the *A. californica* NPV, for the control of the beet armyworm and cabbage looper on cotton, broccoli, and ornamentals. The second contains the NPV from *Spodoptera sunia* and is used on cotton for the control of *S. sunia* and beet armyworm. In France the NPV from *M. brassicae* is sold to control *Mamestra*, *Heliothis*, and *Diparopis* spp. on cotton, cabbage, and other vegetables. In England and Finland the NPV from *N. sertifer* is sold for control of that insect in forests (*47*).

Most of the agrochemical companies in North America have not shown interest in the insect or *in vivo* production system. Sandoz Inc. has developed the only facility specifically for *in vivo* virus production in the United States. In 1980 their commercial *H. zea* NPV, Elcar, was marketed at $7.80 per ha treatment (*48*). However production was discontinued due to low sales in the early 1980's and no viruses are sold in the United States presently. Crop Genetics International of has recently begun development of a facility to produce at least three NPV for control of agriculture and forest pests.

The production of viruses in cell culture is attractive to industry because the process is highly automated and easily controlled. A number of cell lines have been established and the NPVs infecting the major pest insects can be grown *in vitro*. However, the fragility of insect cells and their high oxygen requirements discouraged research on culture systems that would scale up to the volumes needed for virus production. In cultures of three liters or more the increased mixing and sparging required to maintain the required level of dissolved oxygen caused sever damage to the cells. Foaming was shown to be a cause of much of the damage and cell viability in foam was very low (*49*). The bursting of the foam bubbles at the surface of the cell suspension was more damaging to the insect cells than the velocity at which the bubble rose through the suspension. Miltenburger and David (*50*) had avoided this problem in earlier studies by supplying oxygen through silicon rubber tubing coiled around the metal tube of the fermenter's heating system. This enabled them to culture cells in volumes up to 10 liters, but the system was not practical for the larger volumes needed for virus production. Infection of the cells with an NPV further increased the O_2 consumption and virus replication in cultured cells was limited to volumes of 3 liters or less (*51, 52*).

At this time much of the work on large-scale insect cell culture stopped because solutions to these problems were not apparent. The recent discovery of methods for producing monoclonal antibodies from cultured cells led to the development of systems designed to grow animal cells rather than microorganisms. Fermenter vessels were designed with round bottoms and marine type propellers instead of the flat bottoms and blades in microbial fermenters. These new designs were quickly adapted for use with insect cells (*53, 54*). Weiss et al. (*55*) have grown the SF9 cells from *Spodoptera* in 40 liter airlift reactors. Parameters established for optimum cell growth at this volume should apply to cell growth in larger volumes as well.

Another factor limiting the use of cell cultures for virus production was the high cost of media due primarily to the need for fetal bovine serum. This was partially overcome when it was shown that commercial serum replacements could be used in place of the fetal bovine serum and that efficacious virus could be produced (*56, 57*).

Media were then developed in which insect cells grew to high titers without any serum components (*58*). Recently it was shown that NPV produced in cells grown in this type of medium were as infectious for the test insect as were polyhedra produced in insect larvae (*59*). The medium used in this study was projected to cost between $3-5 per liter. Assuming that the cost of materials is 33% of the production costs, viruses could be produced in a bioreactor for $9-15 dollars per liter. Current yields are about 5×10^{10} polyhedra per liter. If the treatment rate is 4×10^{10} polyhedra per ha, the cost would be $8-12 per ha. Thus, it appears that there is now technology for the *in vitro* production of some insect viruses.

Patent Protection. Newly developed selective chemicals can be patented to protect the developer and allow time to recover the investment made in discovery. Naturally occurring microbials have not been patentable and developing companies have not had the advantage of patent protection in the past. However, recent success in patenting both strains of *B. thuringiensis* (U.S. Patent No. 4,950,471) and of the viruses from the celery looper (U. S. Patent No. 4,911,913) and the gypsy moth (U.S. Patent No. 5,124,149) indicate that under some conditions such strains are likely to be protected by patent. Certainly the recombinant DNA strains will have patentability.

Conclusions

A few viruses have become valuable tools in the management of insect pests. There are many more that could be developed in the future. Successful development will require identifying the unique market niche in which the virus will fit, and developing this niche carefully. It is necessary to develop use strategies that take advantage of the attributes of the virus, which are safety, and compatibility with other control measures. A solid data base defining the application rates and the time of application needed to assure consistent control must be established. The promotion of the virus must include the education of all involved with the sale and use to understand how the virus works, and what effects to expect, both in the sort term and in the long term. If this is done viruses will become useful tools in the control of insect pests.

Literature Cited

1. Huber, J. In *The Biology of Baculoviruses Vol. II Practical Application for Insect Control*; Granados, R. R.; Federici, B. A., Eds.; CRC Press: Boca Raton, Florida; **1986**, pp 181-202.
2. Bird, F. T.; Burk, J. M. *Can. Entomol.* **1961**, *93*, 228-238.
3. Lewis, F.B., In *The Gypsy Moth: Research Toward Integrated Pest Management*; Doane, C. C.; McManus, M. L. Eds.; Technical Bulletin 1584, Forest Service, Science and Education Agency: Washington, D. C., **1981**, pp 454-461.
4. Heimpel, A. M.; Thomas, E. D.; Adams, J. R.; Smith, L. *J. Environ. Entomol.* **1973**, *2*, 72-75.
5. *A Catalog of Viral Diseases of Insects, Mites and Ticks*; Martignoni, M. E.; Iwai, P. J. Eds.; General Technical Report PNW-195; Pacific Northwest Research Station, United States department of Agriculture: Washington, D.C., **1986**; 4th Edition Revised.
6. Langridge, W. H. R.; Oma, E.; Henry, J. E. *J. Invertebr. Pathol.* **1983**, *42*, 327-333.
7. Bird, F. T.; Sanders, C. J.; Burke, J. M. *J. Invertebr. Pathol.* **1971**, *18*, 159-161.
8. Milner, R. J.; Lutton, G. G. *Entomophaga* **1975**, *20*, 213-220.
9. Henry, J. E.; Nelson, B. P.; Jutila, J. W. *J. Virol.* **1969**, *3*, 605-610.
10. Classification and Nomenclature of Viruses, Fifth Report of the International Committee on Taxonomy of Viruses; Francki, R. I. B.; Fauquet, C. M.; Knudson, D. L.; Brown, F. Eds.; *Archives of Virology Suppl.* 2; Springer-Verlag: Wein, Austria, **1991**.
11. Katagiri, K.; In *Microbial Control of Pests and Plant Diseases 1970-1980*; Burges, H. D. Ed.; Academic Press: New York, NY, **1981**, pp 433-440.
12. Couch, J. A.; Summers, M. D.; Courtney, L. *Ann. N. Y. Acad. Sci.* **1975**, *266*, 528-536.
13. Wood, H. A.; Granados, R. R. In *Annual Review Of Microbiology*; Ornston, L. N.; Balows, A.; Greenberg, E. P.; Annual Reviews Inc.: Palo Alto, CA, **1991**, *44*, 69-87.
14. Payne, C. C. In *Biological Plant and Health Protection*; Franz, J. M.; Ed.; C. Fisher Verlag: Stuttgart, Germany, **1986**, pp 183-200.
15. Groner, A. In *The Biology of Baculoviruses Vol. I Biological Properties and Molecular Biology*; Granados, R. R.; Federici, B. A. Eds. CRC Press: Boca Raton, FL, **1986**, pp 177-202.
16. Ignoffo, C. M. In *Baculoviruses for Insect Pest Control: Safety Considerations*; Summers, M. D.; Engler, E. R.; Falcon, L. A.; Vail, P. V. Eds. American Society for Microbiology: Washington, DC, **1975**, pp 52-57.
17. Yearian, W. C.; Young, S. Y. In *Microbial and Viral Pesticides*; Kurstak, E. Ed.; Marcel Dekker: New York, NY, **1982**, pp 387-423.
18. Entwistle, P. F.; Evans, H. F. In *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*; Kerkut,G. A.; Gilbert, L. I. Eds. Pergamon Press: Oxford, UK, **1985**, *Vol 12*; pp 347-412.

19. McEwen, F. L.; Hervey, G. E. *J. Econ. Entomol.* **1958**, *51*, 626-631.

20. Vail, P. V.; Whitaker, T,; Toba, H.; Kishiba, A. N. *J. Econ. Entomol.* **1971**, *64*, 1132-1135.

21. Hostetter, D. L.; Biever, K. D.; Heimpel, A. M.; Ignoffo, C. M. *J. Econ. Entomol.* **1979**, *72*, 371-373.

22. Fox, C. J. S.; Jaques, R. P. Can. *J. Plant Sci.* **1966**, *46*, 497-499.

23. Ferre, J.; Real, M. D.; Vanrie, J.; Jansens, S.; Peferoen, M. *Proc. Nat. Acad. Sci. USA*, **1991**, *88*, 5119-5123.

24. Vail, P. V.; Hunter, D. K.; Jay, D. L. *J. Invertebr. Pathol.* **1972**, *20*, 216-217.

25. Asayama, T.; Osaki, J. *J. Invertebr. Pathol.* **1970**, *15*, 284-286.

26. Padmavathamma, K.; Veeresh, G. K. *Entomol. Exp. Appl.* **1991**, *60*, 39-42.

27. Falcon, L. A.; Kane, W. R.; Bethell, R. S. *J. Econ. Entomol.* **1968**, *61*, 1208-1213.

28. Tanada, Y. *J. Invertebr. Pathol.* **1964**, *6*, 378-380.

29. Falcon, L. A. In *Pesticides and Alternatives*; Casida, J. E. Ed. Elsevier Science Publishers B. V. (Biomedical Division): County Clare, Ireland **1990**, pp 109-116.

30. Ignoffo, C. M. *Exp. Parasitol.* **1973**, *33*, 380-406.

31. Fernandez, A. T.; Graham, N. M.; Lukafahr, M. J.; Bullock, H. R.; Hernandez, N. S. *J. Econ. Entomol.* **1969**, *62*, 173-177.

32. Andrews, G. L.; Harris, F. A.; Sikorowski, P. P.; McLaughin, R. E. *J. Econ. Entomol.* **1975**, *68*, 87-90.

33. Bell, M. R.; Romine, C. L. *J. Entomol. Sci.* **1985**, *20*, 146-151.

34. Jones, K. A. In *Pesticides and Alternatives*; Casida, J. E. Ed. Elsevier Science Publishers B. V. (Biomedical Division): County Clare, Ireland, **1990**, pp 131-142.

35. Moscardi, F. Mem. Inst. Oswaldo Cruz, Rio de Janeiro, **1989** *Vol. 84* Supl. III, 51-56.

36. Shapiro, M.; Dougherty, E. M. In *Proceedings Symposium: Microbial Control of Spruce Budworms and Gypsy Moths*; Northeastern Forest Experiment Station, Forest Service, U. S. Department of Agriculture: Broomhall, PA, **1985**, pp 115-122.

37. Shapiro, M.; Dougherty, E. M.; Hamm, J. J. U. S. Patent Appl. Ser. No. 07/609,848 *Composition and Methods for Biocontrol Using Fluorescent Brighteners*. U. S. Patent and Trademark Office, Washington DC

38. Jutsum, A. R. *Phil. Trans. R. Soc. Lond.* B. **1988**, *318*, 357-373.

39. Reinecke, P. In *Pesticides and Alternatives*; Casida, J. E. Ed.; Elsevier Science Publishers B. V. (Biomedical Division): County Clare, Ireland, **1990**, pp 99-108.

40. Hammock, B. D.; Bonning, B. C.; Possee, R. D.; Hanzlik, T. N.; Maeda, S. *Nature* **1991**, *344*, 458-461.

41. Maeda, S. *Biochem. Biophys. Res. Comm.* **1989**, *165*, 1177-1183.

42. Tomalski, M. D.; Miller, L. K. *Nature* **1991**, *352*, 82-85.

43. Maeda, S.; Volrath, S. L.; Hanzlik, T. N.; Harper, S. A.; Majima, K.; Maddox, D. W.; Hammock, B. D.; Fowler, E. *Virology,* **1991**, *184*, 777-780.

44. Stewart, L. M. D.; Hirst, M.; Ferber, M. L.; Merryweather, A. T.; Cayley, P. J.; Possee, D. D. *Nature*, **1991**, *352*, 85-88.

45. McCutchen, B. F.; Choudary, P. V.; Crenshaw, R.; Maddox, D.; Kamita, S. G.; Palekar, N.; Volrath, S.; Fowler, E.; Hammock, B. D.; Maeda, S. *Bio/Technology* **1991**, *9*, 848-852.

46. Cunningham, J. C. *Outlook on Agriculture*. **1988**, *17*, 10-17.

47. Payne, C. C. *Phil. Trans. R. Soc. Lond.* B **1988**, *318*, 225-248.

48. Ignoffo, C. M.; Couch, T. L. In *Microbial Control of Pests and Plant Diseases 1970-1980*; Burges, H. D. Ed.; Academic Press: New York, NY, **1981**, pp 329-362.

49. Tramper, J.; Williams, J. B.; Joustra, D. *Enzyme Microb. Technol.* **1986**, *8*, 33-36.

50. Miltenburger, H. G.; David, D. *Develop. Biol. Standard.* **1980**, *46*, 183-186.

51. Streett, D. A.; Hink, W. F. *J. Invertebr. Pathol.* **1978**, *32*, 112-113.

52. Weiss, S. A.; Orr, T.; Smith, G. C.; Kalter, S. S.; Vaughn, J. L.; Dougherty, E. M. *Biotech. Bioengin.* **1982**, XXIV, 1145-1154.

53. Vaughn, J. L.; Weiss, S. A. In *Large-scale Mammalian Cell Culture Technology*; Lubiniecki, A.S., Ed.; Marcel Dekker: New York, NY, **1990**, pp 597-618.

54. Kompier, R.; Tramper, J.; Vlak, J. *Biotechnol. Letters* **1988**, *10*, 849-854.

55. Weiss, S. A.; DeGiovanni, A. M.; Goodwin, G. P.; Kohler, J. P. **1988**, In *Biotechnol., Biolog. Pesticides and Non Plant-pest Resistance for Insect Pest Management*; Roberts, D. W.; Granados, R.R. Eds. Boyce Thompson Institute for Plant Research, Cornell University, Ithaca, NY **1988**, pp 22-30.

56. Vaughn, J. L.; Fan, F. *In Vitro Cell. Devel. Biol.* **1989**, *25*, 143-145.

57. Vaughn, J. L.; Fan, F.; Dougherty, E. M.; Adams, J. R.; Guzo, D.; McClintock, J. T. *J. Invertebr. Pathol.* **1991**, *58*, 297-304.

58. Vaughn, J. L.; Fan, F. In *Proc. 8th Internat. Conf. Inverteb. Fish Tissue Cult.* Fraser, M. J., Jr. Ed. Columbia, MD, **1991**, pp 111-116.

59. Weiss, S. A.; Godwin, G. P.; Whitford, W. G.; Gorfien, S. F.; Dougherty, E. M. *Proc. 10th Austral. Biotech. Confer. Queensland* **1992**, 67-71.

60. *Atlas of Invertebrate Viruses*; Adams, J. R.; Bonami, J. R., Eds; CRC Press: Boca Raton, FL, *1991*; pp 97.

RECEIVED August 25, 1992

Chapter 18

Development of Improved Bioinsecticides Based on *Bacillus thuringiensis*

Bruce C. Carlton

Ecogen, Inc., 2005 Cabot Boulevard West, Langhorne, PA 19047

Research on the genetics and molecular biology of *Bacillus thuringiensis* (BT) during the past 10-12 years has led to a reasonably clear understanding of the genetic organization and diversity of the insecticidal crystal protein (ICP) genes responsible for the bioactivity of BT strains. This research has led to the recognition that ICP genes are primarily localized to extrachromosomal plasmids, that many strains harbor multiple plasmids for these genes, and that certain of these plasmids are self-transmissible between strains of BT by a conjugation-like process. The molecular cloning and characterization of individual ICP genes has further emphasized the diversity of ICP insecticidal activities. This information has provided both the knowledge base and the methodologies for the systematic construction of new strains of BT having improved properties over their naturally-occurring counterparts. Utilizing both non-recombinant and recombinant genetic approaches, new BT-based products have been developed that are not only superior to older products based on naturally-occurring strains but are competing effectively with synthetic chemical insecticides for many applications.

The development of alternatives to synthetic chemical pesticides is an ever-accelerating activity around the world today. Despite the reassurances from the manufacturers and distributors of chemical pesticides as to their safety, the lesson taught by Rachel Carson in her 1962 book, Silent Spring (*1*) is more than ever apparent. The perception of chemical pesticides by the consuming public is that their safety is questionable, and there is an increasing demand for safer alternatives. Among these alternatives are such approaches as organic farming, employment of farming practices that encourage the control of pests through non-intervention, and the use of biological pesticides. Each of these alternatives has its own merits, and this discussion will concentrate on the latter of these approaches. Of all of the biological agents that have been discovered and evaluated as potential pesticides, very few have achieved a significant penetration into the pest control market in a commercial sense. In fact, of the current annual world-wide pesticide expenditures of greater than

0097–6156/93/0524–0258$06.00/0

$25 billion, less than $150 million (0.6%) represents non-chemical pest control products.

If biologically-derived products are indeed safer, why then have they received so little acceptance within the pest-control marketplace? There are several reasons for this situation, some obvious and some rather subtle. A major historical problem with biological products has been their lack of consistency in field efficacy as compared to their synthetic chemical competition. Over the last fifty years users of chemical pesticides have come to expect high levels of consistency and efficacy in pesticides, and this has not been a strong feature of biologicals. Another major problem with biologicals has been that many of them, especially those based on complex organisms (e.g., fungi, protozoans, and viruses that are obligate parasites of living cells) could not be produced, packaged, and delivered to the user in a cost-effective way. Third, and perhaps less obvious, is that in contrast to the synthetic chemicals, many of which are derived from less effective naturally-occurring progenitor compounds, biologicals have undergone little or no improvement over the years. For example, one of the first biologicals to be marketed commercially in the U.S., *Bacillus popilliae*, a bacterium that generates milky spore disease in white grubs, is still produced today by the same labor intensive *in vivo* production process as it was when first introduced in the late 1940's. Thus a product that potentially could address a $100 million turf insecticide market remains relegated to a sales volume of less than $50 thousand due to the very limited availability of product. Similarly, the expansion of markets for products based on insect viruses has been held back for the same basic reason- the only commercial method currently available for producing the viruses is *in vivo* within the host insects, thus drastically constraining their productivity and cost-competitiveness.

A fourth limitation in the acceptance of biological alternatives to chemical pesticides has been the perceived (and sometimes real) delayed action of biologicals. When a farmer has a problem pest that is endangering his crop, he wants to apply a pesticide that literally stops the pest "in its tracks." Many biologicals, especially those that function as infective agents of pests, require days or even weeks for their effects to be expressed on the pest organism. Insect viruses, for example, must be disassembled in the infected insect target, and then express a complex sequence of functions that may require many days to be fully expressed. During much of this activity by the virus the host insect does not even recognize that it has been infected and thus goes on feeding normally, and continuing to cause crop damage.

For the reasons stated above, biologicals have for the most part received only minor attention as alternatives to chemical pesticides, primarily by environmentally-conscious growers and for home garden and organic farming activities. Probably the one exception to this lack of attention is the bacterial insecticide *Bacillus thuringiensis*, or BT as it is commonly called. BT products have been sold commercially since the late 1950's, and now exceed $125 million in worldwide sales- increasing at the rate of about 20 per cent annually. Part of this expanded usage has resulted from the introduction of new products for different insect applications. The original BT products marketed in the 1950's, '60's, and '70's were only for caterpillar (Lepidopteran) insect control. Following the discovery in 1977 of a new subspecies named subsp. *israelensis*, an array of new products were developed for control of certain Dipteran insects such as mosquitos and blackflies, many of which are important vectors of human diseases such as malaria, Dengue fever, and river blindness disease.

More recently, the discovery in 1982 of the subspecies *tenebrionis* strain active on certain beetle (Coleopteran) insects has produced an array of products directed at Colorado potato beetle on potatoes, eggplant, and tomatoes.

These and other developments at the basic research level have contributed to the general popularity of BT as a bioinsecticide, and the remainder of this chapter will focus on this agent. For an earlier summary of this topic please refer to reference (*2*).

To begin this discussion, a few statements are in order about BT and how it acts as a bioinsecticide . BT is a common gram-positive bacterium, closely related to *B. cereus*, and is found in soils, grain dusts, the bodies of dead insects, and on many plant leaf surfaces. Considerable effort has been put into examining the diversity of BT, and it is now recognized that this bacterial species is indeed very diverse. One criterion that has been used extensively to characterize and categorize BT was developed by de Barjac and associates (*3*) at the Pasteur Institute, and involves the immunological characterization of antigens on surface flagellae; appendages that act as locomotion structures for the bacterium. Currently there are nearly 30 subspecies of BT that are recognized based on this criterion. Another approach to assess the diversity of BT in nature has been described by Baumann *et al* (*4*), who evaluated a wide array of biochemical properties of BT's in an attempt to provide additional criteria for classification. As useful as these approaches have been, neither of them has been definitively correlated with insecticidal activity, which at present can only be determined by *in vivo* bioassay, a very time-consuming and labor intensive procedure. Thus, the characterization of BT strains remains complicated by the different criteria which are used. Regardless of which parameters are used to assess diversity however, the basic conclusion that BT is complex is consistent throughout.

As an insecticide, BT acts as a stomach poison to susceptible insects. As the bacterium grows in culture, it undergoes a pseudo-developmental change resulting in the formation of a spore, which encapsulates the essential genetic information of the cell. When this process (sporulation) is complete the "mother cell" dies and disintegrates, releasing the mature spore into the surrounding medium. The major property that distinguishes BT from its close Bacillus relatives is the production, in sporulating cells, of a prominent proteinaceous crystal, termed the parasporal body. This structure contains most of the insecticidal activity of BT, in the form of insecticidal crystal proteins, or ICP's as they are commonly referred to. When eaten by a susceptible insect, the parasporal body is first solubilized in the insect midgut. Then, the crystalline protein which usually, although not always, exists in the form of an inactive protoxin is converted into an active toxin by a proteolytic activation process. The active toxin binds to either specific or non-specific receptors on the insect mid-guts, and an ion chamnel imbalance is created which ultimately leads to swelling and bursting of the mid-gut epithelial cells. In some insects, the movement of the spore into the hemolymph leads to a secondary septicemia which contributes to the pathogenic response. Recently there has been a great deal of interest in the nature and specificity of the toxin receptors, particularly with regard to the management of the potential for insects to develop resistance to BT toxins. At the moment what is known about this situation is that there are apparently different receptors for BT toxins in given insects, and that different toxins may bind to the same or different receptors (*5*). The next year or two will undoubtedly reveal many new and important aspects of this situation as ongoing studies on the nature of these receptors are completed and reported in the literature.

The diversity of BT has also been well-documented with respect to the various types of different toxin proteins that can be expressed in BT, and the fact that many strains of BT are capable of producing multiple toxin proteins. This situation has served to complicate the understanding of the activity diversity of BT, since a given strain may be expressing widely-varying levels of several different ICP's, each with its own unique activity. The cloning and characterization of individual genes, for which some 25 or so have now been reported, has helped to clarify this situation. Two significant conclusions can be drawn from the information obtained. First is that even very closely-related ICP's may have widely different levels of activity on different insects, even those in the same genus. Second, the activity of a specific ICP varies over a range of as much as 1000-fold on different insects. Both of these observations undoubtedly reflect the specificity of the mode of action of the ICP proteins, which at this writing cannot yet be pinpointed to a specific region or site on the ICP protein.

The ICP proteins can be grouped into families that are closely related in actual amino acid sequence and with respect to insecticidal specificity. Based on these criteria a BT toxin classification system was recently proposed by Hofte and Whiteley (*6*) that allows the placement of ICP proteins into the classification system. For example, the Cry I proteins are grouped together from two perspectives; they all are of the 130-145 kd size range, they are all primarily active only on Lepidoptera and they all have at least 50% identity at the level of amino acid sequence homology. Cry II proteins, in contrast, are much smaller (70-75 kd), some (e.g., Cry IIA) have bifunctional activities on Lepidopterans and Dipterans, and their sequence homology to Cry I proteins is very low. Cry III proteins are those having Coleopteran insect activity, are very similar in size to the Cry II proteins, and have greater inter-group sequence homology than with proteins in other groups. While there will undoubtedly arise exceptions to the clear assignment of toxin proteins to specific groups, particularly as more and more different insecticidal activities are discovered, the system has nevertheless provided a useful beginning to separate the various proteins in a systematic way.

As both genetic and biochemical techniques have become more sophisticated in recent years, the complexity of naturally-occurring BT strains has been clearly documented. From the earlier indications of Krywienczyk and Angus (*7*) that BT crystals contained different antigenic determinants, it is now very clear that a single strain may contain multiple toxin proteins, either of the same family or of different types. Analyses of purified crystals by techniques such as gel electrophoresis have confirmed that a bipyramidal crystal may be composed of different but related toxin proteins. It has also been shown that a single strain may produce more than one crystal type, as exemplified by the highly-studied strain HD-1, the basis for most existing caterpillar control products. This strain produces a large bipyramidal crystal comprised of three Cry I proteins, and a smaller, cuboidal crystal containing a Cry II protein (reviewed in *2*). Through a combination of protein and genetic analyses, using gene probes to identify various toxin genes, it has been well-documented that many BT isolates are multi-genic for these insecticidal proteins, and in some instances may contain as many as six identifiable genes. What is less-well understood at present is how these multi-genic situations are regulated with respect to the individual gene products. While it is evident that the protein products of closely-related genes are expressed to different levels and that transcriptional regulation plays an important role in differential gene expression (*8*), it is not yet clear whether translational or post-translational factors may also play an important role in the ultimate levels of the various proteins expressed by a given strain.

Over the past several years much effort has been expended in developing an understanding of the genetic organization of BT toxin systems. One of the major conclusions from these efforts is that the genes for these proteins are located, for the most part, not on the essential chromosome but instead on an array of non-essential extrachromosomal plasmids (see reference (*9*) for review). Different strains of BT may harbor anywhere from one or two such plasmids to as many as 12-14 different species. A given strain of BT may contain several such plasmids harboring up to five or six different insecticidal toxin genes, including some localized on the same plasmid. The second important conclusion from BT genetic studies is that many of the toxin-encoding plasmids are capable of self-transmission from one cell to another by a naturally-occurring process called conjugation (*10*). This is a type of sexual gene transfer previously described in great detail for *E. coli* and other bacteria and for which Joshua Lederberg shared the Nobel Prize in Physiology or Medicine. In BT, this process has been observed to permit reciprocal transfer of toxin-encoding plasmids at high efficiency (up to 75%) in matings between different parental strains.

A second property of significance to BT toxin plasmids is their ability to be spontaneously lost ("cured") from cells that harbor them. Variants can be readily recovered that have lost one or more of their plasmid complement, and if the cured plasmid contains an insecticidal toxin gene the resulting mutant strain becomes crystal-negative (unless it harbored another toxin-encoding plasmid). Regardless, there are molecular techniques that can readily distinguish such cured variants. If derivatives are identified that have been cured of toxin-encoding plasmids that contribute relatively little to the activity against a particular insect target, the typical result is that the remaining toxin gene products are expressed in relatively higher amounts. This is because there is an apparent built-in dosage compensation effect that allows the production of a certain level of toxin protein, within some as yet undefined range of ICP genes. Although this regulatory system is not yet well understood, the data from various mutant strains are sufficiently convincing that this conclusion appears valid.

Thus, one way of improving a naturally-occurring BT strain is to isolate cured variants that have lost plasmids encoding inactive or poorly-active toxins. A variation of this approach that exploits the natural transmissibility of BT toxin plasmids involves the mating of a partially-cured variant of an otherwise desirable strain with a second strain having a toxin plasmid with some desired property. The resulting transconjugants will typically yield some derivatives which have acquired the desired toxin plasmid from the donor strain, effectively replacing the undesired or poor activity with a better activity. Depending upon how many toxin plasmids were originally present, this first transconjugant may be mated with still another plasmid donor strain to transfer in another toxin plasmid. This process allows the accumulation, in a single strain background, of an array of BT toxin plasmids derived from several different parental origins. By the selective choice of parental donor strains possessing either more potent insecticidal activities, or different insecticidal activities, new strains can be constructed that are more active or that have broader target insect spectra. In addition, the properties of such genetically-derived strains provide the basis for the filing of patents to provide a means of proprietary protection.

Development of Improved BT Products by Non-Recombinant Approaches

Based upon the foregoing considerations Ecogen Inc. set about several years ago on a BT improvement program. This program was designed with several integrated components to ensure the most rapid development of new BT-based products.

First was an aggressive new strain discovery program to expand its library of BT insecticidal activities. Samples were collected from all over the world and carefully examined to identify new and different strains of BT, using a variety of criteria such as plasmid arrays, insecticidal activities, toxin gene profiles, etc. Through these activities we have amassed a collection of over 8000 novel BT isolates having a broad array of characteristics. From the strains identified as having desired characteristics, a strain development program was instituted to utilize the procedures of plasmid curing and conjugal transfer described previously to generate new gene combinations directed at specific crop and insect applications. From many hundreds of such constructs a small number of candidate strains was identified with the desired levels of activity and/or insecticidal spectrum. These strains were subjected to an intensive evaluation program, including fermentation process behavior, greenhouse performance in on-plant assays, formulation behavior, and field performance. From among those having acceptable properties were selected three strains of different genetic compositions for development as new products. One of these products, Condor® bioinsecticide, was specifically designed for improved performance on soybean caterpillars, cotton caterpillars (the so-called *Heliothis* complex), and caterpillars that attack forest trees (gypsy moths and spruce budworms). Condor also has excellent performance against an important vegetable insect pest, the diamondback moth. A second product, Cutlass® bioinsecticide, was developed for improved efficacy against a broad array of vegetable crop pests, including cabbage loopers, beet armyworms, webworms, imported cabbageworms, and others. Although the BT strains contained in the two products are genetically distinct, the nature of the gene compositions in the two strains provides for significant overlap in their insecticidal activities. The third product, Foil® bioinsecticide is very different in its activity spectrum, however, since it combines in a single strain activities against both beetle and caterpillar insects. This is a truly novel construct that we have not observed in natural BT isolates, and is the only product of its type that has ever been commercialized to our knowledge. Foil® was developed to control a complex of beetle and caterpillar pests on crops such as potato, tomato, and eggplant. U.S. patents have been received on all three of these strains and are pending in foreign countries. Condor®, Cutlass®, and Foil® are all fully-registered for sale in the United States, and registrations are currently being pursued in other countries. It is important to note in the context of the registration of BT products that the U.S. Environmental Protection Agency, which is responsible for the regulatory oversight and approval for sale of all pesticide products in the U.S., has determined that genetically-altered strains of BT generated by plasmid curing or conjugal transfer are considered the same as naturally-occurring strains with respect to risk assessment and environmental impact considerations. Obtaining registrations for new BT products developed by these techniques has thus been greatly facilitated. The registrations for Condor®, Cutlass® and Foil® were received following only a few months in the review process.

Development of Improved BT Products By Recombinant Approaches

Despite the advances in BT strain development that have been possible through the non-recombinant methods described, there are significant limitations to the genetic improvements that can be made with these approaches. For example, the conjugal transfer approach requires that the gene to be transferred be located on a transmissible plasmid. Although many BT plasmids are capable of transfer, either on their own or through mobilization by other plasmids, some are very difficult to move from one cell to another or do not transfer at all. A second limitation of the non-recombinant approaches is a consequence of the frequent localization of two or more insecticidal genes on the same plasmid. If one of these is a desired gene and the other encodes a poorly-active protein, the curing or conjugal transfer techniques only allows moving the two genes together. Thus, for greater flexibility in new strain construction it is highly desired that techniques be available to transfer any gene from any donor source into any desired recipient. Such a technique is obvious through the use of recombinant DNA, or enzyme-mediated gene splicing.

In choosing a strategy for BT product improvement utilizing recombinant DNA techniques, we elected to utilize, as the expression and production system, the BT bacterium itself, for several reasons. First is that BT is already a very efficient production system. BT cells synthesize typically greater than 30 per cent of their cell mass in the form of the insecticidal crystal proteins, and have clearly evolved very efficient expression systems for these proteins. Second, was the objective of developing products containing multiple insecticidal activities, much as naturally-occurring BT strains typically have. Since BT is known to maintain in a genetically-stable way such multigenic situations, we believe that the choice of BT as the host organism will minimize stability problems in the future.

The objective of multigenic constructs has three significant advantages. First is that these constructs allow one to take advantage of the potency synergies that frequently occur between different insecticidal proteins. In addition, the use of multigenic strains having activities with different receptor binding properties or other aspects of their mode of action helps to minimize the potential for the target insects to develop resistance to the products. Lastly, the choice of BT as an expression host for the generation of new insecticidal gene combinations we believe will create less of a regulatory concern than if these activities were expressed in a heterologous host.

For a cloning vector, we chose to isolate replication origins from native BT plasmids. This choice was based on two considerations. First was to maintain an all-BT nature of the recombinant constructs. Second was that we reasoned that native BT plasmids, particularly those that naturally harbor insecticidal toxin genes, should be stable when such genes are spliced onto them. From these considerations a number of replication/maintenance fragments were initially cloned from a BT strain of subspecies *kurstaki*. It turned out in subsequent analyses that these replication fragments had a number of very interesting properties. First, of the three replication origins derived from a single BT parent, all had extremely different nucleotide sequences (which presumably explains why they could naturally co-exist within the same cell). Second, although two of these origins were derived from plasmids capable of high efficiency transfer by conjugation, the replication fragments derived from them had drastically reduced transfer efficiencies, either by themselves or in the presence of mobilizing plasmids. Third, when many different BT strains were probed with these replication origins it was observed that they were ubiquitous, in that one or more were found to exist in many of the strains examined. For each of these replication origins we constructed shuttle cloning vectors by adding an *E. coli* replicon, a Gram-positive antibiotic selection marker that is expressed in BT, and a small synthetic fragment of DNA containing a number of unique restriction enzyme cleavage sites that serves as a multi-insertion site for a variety of different ICP genes. These vectors are uniquely constructed such that either the *E. coli* DNA or the antibiotic resistance marker, or both, can be conveniently removed by cleavage of the shuttle vector with specific restriction enzymes whose sites are not present within the BT replication fragment or the ICP genes to be cloned. Thus, with this cloning system, initial cloning and characterization of ICP genes can be done in *E. coli*, which has a number of important advantages. The desired clones can then be transferred to BT, in which their stability can be examined both in the presence and absence of the selected antibiotic. Ultimately, for a final product the recombinant plasmid can be finally modified to remove all of the foreign DNA, leaving only the BT replication origin, the cloned ICP gene, and the small amount of synthetic DNA from the multi-cloning site (see reference *(11)* for a review of this cloning and expression system).

Utilizing this system, we have constructed recombinant strains with improved potencies and increased fermentation productivities. One of the prime examples of this approach is illustrated by a derivative of our current Foil® product that was field-tested both in 1991 and in 1992. The original Foil® product contains a strain that, by the conjugal transfer system, has two copies of a caterpillar-active gene and one copy of a beetle-active gene. During fermentation, the strain produces roughly two-thirds of the insecticidal toxin of caterpillar type and one-third of the beetle toxin. We inserted into this strain a recombinant plasmid containing a new beetle-active toxin gene that we isolated on one of our proprietary plasmid vectors. Due to the fact that the original Foil strain carried a toxin-encoding plasmid having the same replication origin as that of the recombinant plasmid, transformants were recovered in which one of the native plasmids containing a caterpillar-active toxin gene was replaced by the related replication origin of the recombinant plasmid containing the novel beetle-active gene. Thus the recombinant derivative now contains two different beetle-active genes, and only one caterpillar-active gene. During fermentation this switching of gene ratios is basically reflected in a similar reversal of the relative amounts of production of the beetle-active and caterpillar-active proteins. This new recombinant (EG7618) was field-evaluated in 1991 at two locations and showed excellent performance in control of Colorado potato beetle (caterpillar pest control was not evaluated in these trials). More extensive field trials are being conducted as this is being written. The importance of this recombinant strain is two-fold. First, by changing the gene ratios of the original Foil® strain we have essentially achieved a two-fold increase in the production of the beetle-active protein per volume of fermentation broth. Second, since the added beetle-active gene is different than the one originally present in Foil®, it has certain important differences in the activity of its ICP toxin on young adult larvae of Colorado potato beetle, thus offering a potentially important advantage over similar products that lack adulticide activity (see reference (*12*) for a more complete description of this strain).

Similar constructs utilizing a variety of new gene combinations are currently under evaluation against other important target insects. It is our expectation that the additional advantages of this BT-based plasmid vector system, employed in concert with our strain discovery program, will allow us to make significant and dramatic improvements in BT-based insecticide products within the very near future, and we are optimistic in obtaining permission to field-test such strains and to register them for commercial production. It is our firm belief that the future is extremely promising for a broad new array of BT-based insect control products, that will not only be highly effective but will be both cost-competitive with current chemical insecticides and much less likely to cause problems with resistance.

Acknowledgments

The author thanks Dr. Cynthia Gawron-Burke for critical review of this manuscript, and Ms. Lee Radke for expert assistance in its preparation.

LITERATURE CITED

1. Carson, R. *Silent Spring*, Houghton-Mifflin, Boston, 1962.
2. Carlton, B. In *Biotechnology for Crop Protection;* Hedin, P.A., Menn, J.J., and Hollingworth, R. M. Eds., ACS Symposium Series 379; 1988 Chap 19, p. 260-279.
3. de Barjac, H. In *Microbial Control of Pests and Plant Diseases,* 1970-1980; Burges, D.. Ed., Academic Press: New York, 1981, Chapter 3, pp.35-43.
4. Baumann, L.; Okamoto, K.; Unterman, B.; Lynch, M.J.; Baumann, P. *J. Inv. Path.* 1984, *44*, 329-341.
5. Hofmann, C.; Luthy, P.; Hutter, R.; Pliska, V. *Eur. J. Biochem.* 1988, *173*, 85-91.
6. Hofte, H.; Whiteley, H. *Microbiol. Rev.* 1989, *53*, 242-255.
7. Krywienczyk, J.; Angus, T. A.; *J. Inv. Path.* 1967, *9*, 126-128.
8. Arvidson, H.; Dunn, P. E.; Strnad, S.; Aronson, A. I. *Molec. Microbiol.* 1989, *3*, 1533-1543.
9. Carlton, B. C.; Gonzalez, J. M., Jr. In *The Molecular Biology of the Bacilli, Vol. II*; Dubnau, D. A., Ed.; Academic Press: New York. 1985; Chapter 8, pp. 211-249.
10. Gonzalez, J. M., Jr.; Carlton, B. C. In *Genetic Exchange: A Celebration and a New Generation;* Streips, U. N., *et al*, Eds.; Marcel Dekker: New York, 1982; pp. 85-95.
11. Gawron-Burke, C.; Baum, J. A. In *Genetic Engineering, Vol. 13;* Setlow, J. K., Ed.; Plenum Press: New York, 1991; pp. 237-263.
12. Gawron-Burke, C.; Johnson, T. B. In *Advances in Potato Pest Biology and Management;* Zehnder, G., Ed.; APS Press: St. Paul, *in press*, 1992.

RECEIVED September 25, 1992

Chapter 19

Use of *Bacillus thuringiensis* Genes in Transgenic Cotton To Control Lepidopterous Insects

Johnie N. Jenkins

**Crop Science Research Laboratory, Agricultural Research Service,
U.S. Department of Agriculture, Mississippi State, MS 39762**

Transgenic cotton, Gossypium hirsutum L., plants containing modified, truncated versions of the cryIA genes from Bacillus thuringiensis var kurstaki are resistant to many Lepidopterous insects. Plants containing specific, truncated versions of the cryIA gene were resistant to tobacco budworm, Heliothis virescens Fab., in field and laboratory studies in my laboratory. Other researchers have shown that these transgenic plants are also resistant to bollworm, Helicoverpa zea (Boddie); pink bollworm, Pectinophera gossypiella (Saunders); cotton leafperforator, Bucculatrix thurberiella Busck; and saltmarsh caterpillar, Estigmene acrea (Drury). Others have shown that the proteins coded for by the B. thuringiensis genes bind selectively to sites in the brush borders of epithelial cells in the midgut of the insect and induce the formation of small pores (0.5 to 1.0 nm) in the cell membranes, resulting in a net influx of ions and outflow of water. The cells swell and lyse. The B. thuringiensis genes in transgenic cotton plants appear to offer an environmentally desirable control method for Lepidopterous cotton pests when used in integrated pest management programs.

Introduction to Host Plant Resistance

Pest resistant cultivars are the most environmentally benign method available for control of many crop pests. For many pests they are the only method available. The cultivar forms the foundation upon which successful integrated pest management programs are built. Resistance genes are available in crops for insects, mites, nematodes, and plant diseases caused by fungi, bacteria, and viruses; however, cultivars are not usually resistant to all pests that affect the crop.

There are several advantages of resistant cultivars: 1) pest control is bought when the seed are purchased, as the control is bred into the seed; 2) this method of pest control is compatible with most other methods of control; 3) resistant cultivars form a foundation upon which integrated pest management programs can be built; 4) few or no adverse effects on the environment are caused

This chapter not subject to U.S. copyright
Published 1993 American Chemical Society

by the genes for resistance; 5) cost of this type of pest control is usually minimal; and 6) compared to conventional chemical pest control, less effort is required after the seeds of resistant cultivars are planted; however, for cotton, Gossypium hirsutum L., integrated pest management involving control of several pests will be required.

Host plant resistance, i.e., the development of resistant cultivars, is a dynamic process. Most insect pests and pathogens of important crop plants are very diverse and have a wide host and/or geographic range. These pests are dynamic and their population makeup is subject to selection pressure from host cultivars and other factors in the environment. A resistant cultivar can exert selection pressure upon a variable population of insects and, thus may select for a biotype that can successfully colonize and damage the cultivar. The degree of selection pressure strongly influences how long a gene or set of genes will be useful for control of a particular pest. Some genes for resistance to pests last for many years; whereas, others last only a few years. The most dramatic examples of successful control of insects with resistant cultivars are the control of hessian fly, Mayetiola destructor (Say), in wheat, Triticum spp.; and brown planthopper, Nilaparvata lugens, in rice, Oryza sativa (1).

Twenty genes for resistance to hessian fly have been discovered (2). During the period from 1950 to 1983, 60 cultivars of wheat with various combinations of these genes for resistance to hessian fly were developed and successfully used to control this pest (2). There is great variability in the length of time a particular gene is useful for pest control before the selection and development of a pest biotype that is resistant to the gene occurs. For example, the first gene for resistance to brown planthopper was Bph-1 and was in the cultivar IR26 released in 1973 (3). Widely planted in the Philippines, Indonesia, and Vietnam this cultivar became susceptible to brown planthopper in just three years after being released (1). This gene was effective but only for a relatively short time, yet it was very successful during this time.

Plant breeders continued to work and developed rice cultivars with a different gene for resistance to the new biotype of the brown planthopper. IR36 and IR38 with the bph-2 gene were developed and released (4) and became the dominant rice cultivar in many areas. This source of resistance has held up for 14 years and is still useful in many areas (1). Continuous research on cultivar development, discovery, and identification of new genes for resistance to brown planthopper has resulted in the breeding and release of cultivars with additional genes for resistance. This effort has culminated in the release of IR56 and IR60 with the Bph-3 gene for resistance which was useful when a resistant biotype was discovered in small pockets of the Phillipines and Indonesia. IR66 with the bph-4 gene for resistance was released in 1987 and IR68, IR70, IR72, and IR74, all with the bph-4 gene for resistance, were released in 1988. These cultivars are widely grown in tropical and subtropical rice growing countries of the world (1). These developments illustrate the continuous effort necessary to successfully utilize host plant resistance for pest control. Utilizing genes made available through recombinant DNA technology probably will not change this. Many factors influence how long a particular gene for resistance will be useful. These examples, however, illustrate that it is possible to develop resistant cultivars for pest control that are friendly to the environment; safe, efficient, and economical for the grower; and useful to the consumer.

This chapter reviews current research involving transgenic plants for insect control. It deals primarily with cotton and genes from Bacillus thuringiensis because the majority of research with transgenic plants for insect control involves genes from this bacterium and cotton will probably be the first crop to be grown commercially with these genes.

Conventional Plant Breeding

Conventional plant breeding involves crossing plants, usually within a species, and transferring genes for useful traits. Genes that confer pest resistance are often found in unadapted, low yielding land races of crop plants or in weedy relatives of crop plants. An arduous and long process is necessary before an adapted cultivar can be developed with these gene(s) for resistance. This process has worked very well because plant breeders and associated scientists have realized that resistant cultivars are useful and that pest population gene pools are dynamic and influenced greatly by resistant cultivars. Their continuing work has resulted in discovery of new genes for resistance and the deployment of these through new cultivars resistant to pests. Species barriers have been major obstacles to this type of research. Plant breeders have been more successful with intraspecific crosses. Only a few examples exist where genes from other closely related species have been successfully utilized in breeding programs. Fertile crosses are usually limited to plants within the same species and some closely related relatives. These have been the constraints within which plant scientists have worked. Modern cultivars of cotton show that plant breeders have been successful in developing cultivars with higher yields and higher effective levels of pest resistance (5).

Biotechnology and Recombinant DNA Technology

The developments in recombinant DNA technology and other aspects of biotechnology, including genetic engineering, have improved the opportunities for plant scientists. Genetic engineering complements conventional plant breeding by increasing the diversity of genes available for incorporation into crops. It is now theoretically possible to move a gene from any organism to a plant and have that gene produce its product in the plant. Presently there are limitations upon which types of genes can be moved and upon how many genes can be moved at one time; however, as this technology advances, many of the present obstacles will be erased or reduced. Genetically changed plants, in which genetic material from other species is inserted by biotechnology, are called transgenic plants. Transgenic plants have been developed for 47 species of crop plants (6). These involve all the major food and fiber crops of the world.

Fraley (6) says that, "Between 1992 and 2030 farmers will have to produce more food--more calories--than they have done from the beginning of agriculture until now." He asks and answers the question: "In short, how can we create sustainable agriculture? Invest in, and develop new agricultural technologies." Plant biotechnology is one of these new technologies that will be developed to help meet these future human needs.

Systems for Producing Transgenic Plants

The most advanced systems for producing transgenic cotton plants have involved Agrobacterium tumefaciens as the genetic engineering agent. This bacteria infects many dicotyledonous plants and causes the growth of tumors or galls in the infected plants. Virulent strains of the bacteria contain large Ti (tumor-inducing) plasmids, which are responsible for the DNA transfer and subsequent disease symptoms. These Ti plasmids contain two sets of sequences necessary for gene transfer to the plants. One of these sequences is the T-DNA (transferred DNA) region which is transferred to the plant. The other sequence, the vir (virulence genes), is not transferred during infection. The T-DNA regions are flanked by border sequences that determine the definition of the region transferred to the infected plant. A special type of the bacteria which has the tumor-inducing genes disarmed is used in transformation experiments. Disarming the tumor inducing genes allows the bacteria to infect the plant, but does not allow the formation of tumors. The desired genes that have been inserted between the flanked border sequences of the Ti plasmid are then inserted into the plant by the bacteria in a manner similar to normal infection of the plant in nature. Thus, the infection process of the A. tumefaciens bacteria is used to insert the gene of interest rather than the tumor inducing gene and becomes the vehicle by which foreign genes can be inserted into plants.

A second major means of producing transgenic plants is the particle bombardment process. A recent review of gene transfer by particle bombardment describes how this method is being used with plants and animals, and reports important applications of the process to produce transgenic maize, Zea maize, and soybean, Glycine max, as well as the introduction of DNA into plastids and mitochondria (7). One application is the direct insertion of genes into organs of living animals. This technique is also being used to transform cotton (8, 9). A recent review of biotechnology of cotton summarizes many of these concepts (10).

As plant development is better understood, we are beginning to achieve a better understanding of how gene regulation occurs. Tissue specific promoters and regulators are now available that when linked with pest resistant genes should allow for tissue specific expression of the pest resistance genes (11). Resistance to the herbicide glyphosate has been enhanced by targeting the product of a mutated form of the EPSPS bacterial gene to reside in the chloroplasts of plants where EPSPS normally occurs (12). There are situations where the tissue specific approach would be desirable from a pest control or safety approach. Gasser and Fraley (10) suggest that in the near future we will have on hand a large number of regulatory sequences that will allow for accurate targeting of gene expression in specific tissue within transgenic plants. Reducing the expression of a specific gene in a plant can also be useful. Through anti-sense RNA technology this is now possible and has found application in tomatoes, *Lycopersicon esculentum*, where the messengers for polygalacturonase have been altered with anti-sense RNA. As a result the altered tomatoes do not produce much of this enzyme and the tomatoes ripen without becoming soft (11, 13).

B. thuringiensis Endotoxin for Insect Control

The most widely used genes for insect control in transgenic plants are those from several strains of *Bacillus thuringiensis*. This bacteria produces proteins which are lethal to selected insect pests. Many strains of *B. thuringiensis* are toxic to insects in the order Lepidoptera, some are toxic to insects in the order Dipteria (fly), and some to Coleoptera (beetle). Recently a strain of *B. thuringiensis* has been discovered that is toxic to nematodes (14, 15).

Tobacco, *Nicotiana tabaccum*, plants were first transformed with the *B. thuringiensis* delta endotoxin gene in 1987 (16). Insertion of the 1176 amino acid toxin in plant cells was phytotoxic; however, Barton et al. (16) reported that eliminating the protoxin carboxyterminus solved the phytotoxicity problems and produced tobacco plants that expressed the delta endotoxin.

Commercial cotton cultivars were first transformed using *A. tumefaciens* mediated transformation technology (17). Effective selection for kanamycin resistance in tissue culture, regeneration of plants, and expression of the marker enzymes, neomycin phosphotransferase II (*npt* II) and chloramphenicol acetyltransferase (*cat*), at the whole plant level showed that transformation had occurred. Direct evidence of foreign DNA integration was provided by southern blot hybridization.

The first field test of transgenic cotton plants containing the *B. thuringiensis* gene encoding for the delta endotoxin was conducted in 1989; however, these plants did not provide control of tobacco budworm insects in the field or in the laboratory (18). Subsequent field tests of cotton strains in 1990, using plants with a different form of the *B. thuringiensis* gene, did however, provide effective field control of tobacco budworm (19).

Other Genes for Insect Control in Transgenic Plants

A gene encoding a cowpea, *Vigna unguiculata*, trypsin inhibitor (CpTI) has been shown to confer a level of resistance to tobacco budworm when transferred into tobacco (20). Cowpea trypsin inhibitors are small polypeptides of around 80 amino acids belonging to the Bowman-Birk type of double-headed serine protease inhibitors and are the products of a small gene family (21).

Insecticidal Crystal Proteins Present in *B. thuringiensis*

Nucleotide sequences for 42 crystal protein genes from *B. thuringiensis* have been described (22). Several sequences are nearly identical. There are 14 distinct crystal protein genes of which 13 specify a family of closely related insecticidal proteins (Cry proteins). These are divided into a minimum of four major classes and several subclasses specified by structural similarity and insecticidal spectra of the encoded proteins. The major classes are Lepidoptera specific (I); Lepidoptera and Diptera specific (II); Coleoptera specific (III); and Diptera specific (IV).

The Lepidoptera specific crystal proteins are the best characterized. The 20 different cry I sequences identified, can be divided into six different genes. These are cryIA(a), cryIA(b), cryIA(c), cryIB, cryIC, cryID (22). These have 1176, 1155, 1178, 1207, 1189, and 1165 amino acids, respectively. All 20 genes encode 130 to 140 kDA proteins which are accumulated in bipyramidal crystalline inclusions during sporulation of B. thuringiensis. These proteins are protoxins and are solubilized in the alkaline environment of the insect midgut and are proteolytically converted by crystal associated or larval midgut proteases into toxic core fragments of 60 to 70 kDa.

Strains of B. thuringiensis produce several different crystal proteins simultaneously and the same or very similar crystal proteins occur in strains of different subspecies (22). Most of the genes are located on large conjugative plasmids, thus, mobility of crystal protein genes among subspecies is expected. This mobility property has been exploited to develop strains of B. thuringiensis with desired genes from two or more strains.

Genes encoding for insecticidal proteins for Lepidoptera have been genetically engineered into transgenic cotton plants. The wild type genes are expressed poorly in plants (23). Therefore, various modifications of the genes are needed to increase expression and increase activity toward Lepidoptera insects. For example, plants with a partially modified cryIA(b) gene had a 10-fold higher level of insect control protein than wild plants, and plants with a fully modified cryIA(b) had a 100-fold higher level of the protein (23). Similar results were obtained in plants with the fully modified cryIA(c) gene. Partially modifying the gene involved selectively removing DNA sequences predicted to inhibit efficient plant gene expression without changing the amino acid sequence. Fully modifying the gene involved wholesale changes in DNA which required the use of a fully modified synthetic gene (23). This increased gene expression was generic across several plant species--tomato, cotton, and tobacco. These results show that it is quite possible to modify wild type genes in a manner that is favorable for insect control.

Mode of Action of B. thuringiensis Insect Control Proteins

The general characteristics exhibited by insects upon ingestion of the B. thuringiensis toxin are: 1) cessation of feeding within one hour, 2) reduced activity within 2 hours, and 3) progressive sluggishness and paralysis within 6 hours. The epithelial cells swell with disrupted microvilli, cell lysis, and cell sloughing occur, and the insects die as a combined result of starvation and septisemia. Our experience with transgenic cotton plants with a modified B. thuringiensis gene indicates that neonate tobacco budworm larvae feed on transgenic tissue for only a few bites and then stop. They then become lethargic and die within 3 to 6 days.

The delta endotoxins are synthesized as large protein molecules and crystalized as parasporal inclusions. When susceptible insects ingest the crystals, they are dissolved in the midgut of the insect and protoxins are released that are about 140 kDa in size for the cryI proteins. They may be further processed by the insect to smaller toxic molecules. The toxic protein molecule then binds with specific affinity to receptors in the midgut epithelial cells, where pores or ion channels develop in cell membranes. This disturbs cellular osmotic balance and causes cells to swell and lyse. These effects cause paralysis of the insect midgut and mandibles, and thus, death occurs through a combination of starvation and septicemia. The ultrastructural changes and time course of poisoning varies between insect species and the various cryI toxins.

Limitations to Use of B. thuringiensis Genes

It is well known that pest resistance to conventional insecticides is widespread. We should expect that genetically engineered plants or biopesticides will also be susceptible to the same problems. Conventional forms of B. thuringiensis pesticides have been used for over 20 years without widespread development of resistance; however, in the past few years several instances of resistance have been reported. A tobacco budworm strain has been selected in the laboratory to be 13X to 20X more resistant to diet incorporated Pseudomonas fluorescens which had been genetically engineered to express the 130kDa protein from the HD-1 strain of B. thuringiensis (24). These same insects were 4X less susceptible to purified HD-1 endotoxin and to Dipel^R, which is a commercial formulation of a crystal-spore mixture of B. thuringiensis var kurstaki.

Plodia interpunctella, the Indian meal moth, a pest of stored grain, developed resistance to a commercial formulation of B. thuringiensis when stored grain was treated with B. thuringiensis for a few insect generations (25). Resistance increased nearly 30-fold in two generations in a strain of this insect reared on diet treated with B. thuringiensis and after 15 generations resistance reached a plateau 100 times higher than the control level (25). From these reports, we should expect that insect pests of cotton have the genetic capability to develop resistant biotypes via selection by transgenic cultivars. We should at least recognize that the possibility for resistance development exists and that the potential for resistance varies with the insect and the cultural practices for the crop. This should motivate researchers to develop means of dealing with the potential for resistance.

Mechanisms of Resistance to B. thuringiensis

Studies on a strain of Indian meal moth selected in the laboratory for resistance to a B. thuringiensis insecticidal crystal protein in Dipel, provide evidence that resistance in this insect is due to an alteration in binding of the toxin to cell membranes (26). This research provides insight into how the B. thuringiensis protein is associated with toxicity in the insect. Two distinct changes have apparently occurred in the resistant strain of Indian meal moth. The first change is that this strain is resistant to insect control protein of the CryIA(b) type, but there is no resistance to CryIC protoxin or CryIC toxin. This (CryIC) insect control protein is not present in crystals of Dipel; whereas, the insect control proteins of CryIA(b) type are present in Dipel which was used to select the resistant insects. The second change that has occurred is a marked increased sensitivity to CryIC protoxin and CryIC toxin in the resistant strain (26). Van Rie et al. (26) suggest that when two insect control proteins are available for the same insect, resistance to one insect control protein does not necessarily confer resistance to the second insect control protein; therefore, insect control proteins with different binding properties could be used to delay development of resistance. Research on the molecular mechanisms involved in resistance is expanding rapidly and better ways to manage resistance should emerge from these studies.

The Indian meal moth population studied by Van Rie et al. (26) was selected for resistance in the laboratory. Insect strains selected for resistance in the laboratory may or may not have the identical genes for resistance as those selected under field conditions. In Hawaii, the Diamondback moth, Plutella xylostella (L.), is resistant to the insecticidal spore-crystal protein complex of B. thuringiensis (27). This resistance was developed in response to commercial foliar applications of B. thuringiensis. In the Phillipines a strain of diamondback moth resistant to commercial formulations of Dipel was established from pupae (28). Crystal proteins, from recombinant genes CryIA(b), CryIB, and CryIC, were obtained as proteins expressed in Escherichia coli and the proteins were evaluated for binding to the brush borders of membrane vesicles (BBMVs) of the resistant and a susceptible laboratory strain. All three proteins bound to the BBMVs from the susceptible laboratory strain; however, CryIA(b) protein did not bind to the BBMVs from the resistant strain. These results indicate that the susceptible laboratory strain has specific receptors for CryIA(b), CryIB, and CryIC, whereas in the resistant strain, receptors were only detected for CryIB and CryIC (28). Resistance in the diamondback moth and the Indian meal moth each involved membrane receptor mechanisms, even though one was selected for resistance under field conditions and one under laboratory conditions. Thus, membrane receptor mechanisms are likely to be one of the mechanisms involved in resistance of other insects to B. thuringiensis.

Management Strategies for B. thuringiensis Transgenic Cotton

Various strategies have been proposed for management of B. thuringiensis genes used in cultivars of crops to delay or prevent the development or selection of strains of insects that are no longer susceptible. The strategies must consider that the toxins from B. thuringiensis will be used in the form of transgenic plants as well as in the form of commercial applications of spray B. thuringiensis products. The first commercial transgenic crop to use modified B. thuringiensis genes in the plant for control of insects will probably be cotton. Commercialization will probably occur in the mid 1990's. Industrial companies with transgenic cotton germplasm are developing corporate arrangements with established cotton seed breeding companies that will provide growers with transgenic cultivars of cotton with selected proprietary B. thuringiensis genes.

Promising results have been obtained from field tests conducted for two years with transgenic cotton strains for control of Lepidopterous insects. Resistance management in this crop will employ several techniques not yet fully developed. Several conferences involving Industry, Government, and Academic scientists have considered strategies for management of B. thuringiensis genes; however, no strategies have been agreed upon by all concerned parties.

As one possible means to manage the development of resistance, a system has been devised for temporally controlling the expression of the endotoxin. This has been accomplished in tobacco. Plants containing a toxin gene driven by a chemically-responsive promoter were developed. When treated with a chemical regulator, chemical induction resulted in accumulation of toxin mRNA which caused the plants to become insect tolerant (29). This ability to control when the toxin gene is expressed, should be of value in resistance management.

A general scenario for resistance management in cotton could be as follows:

- A. Short Term (at commercialization):
  1. High dose expression of the gene in cotton plants to control insects heterozygous for resistance alleles.
  2. Refugia as hosts for sensitive insects.
  3. Crop management practices that minimize insect exposure to the gene.
  4. The use of integrated pest management tactics.
  5. Monitoring of insect populations for susceptibility to the gene or genes.
- B. Medium Term (implemented 2 to 5 years after commercialization):
  1. Continue all short term strategies plus:
  2. Combine two B. thuringiensis genes into the same plant, each of which produce toxins that are active on target insects but with different sites of action.
- C. Long Term (more than 5 years after commercialization):
  1. Continue all short and medium term strategies plus:
  2. Incorporate natural cotton host plant resistance genes into the transgenic cotton cultivars as the natural cotton genes are proven effective.
  3. Incorporate non B. thuringiensis proteins to provide effective control of Lepidopterous pests.

There is much planning and research effort involved in developing strategies to successfully utilize transgenic cotton plants. Similar efforts are under way with other crops being considered for transgenic approaches to pest control.

Recent research is providing new prospects for use of various B. thuringiensis products and a wide range of possible uses for this biological pesticide in forms ranging from transgenic plants to newer types of B. thuringiensis spray products with widened ranges of potencies (15). A list of 18 major companies involved with research on various B. thuringiensis products is shown. Five of the companies are involved with B. thuringiensis in plants. Of the 53 U.S. patents on B. thuringiensis granted in the last 21 years, 39 have been issued in the last 4 years (15). These newer products may also be useful in managing resistance to B. thuringiensis endotoxins, including transgenic plants.

Results of Experiments with B. thuringiensis Genes in Cotton

Insect resistant transgenic cotton plants containing B. thuringiensis genes genetically engineered into cotton have been reported (30). Field and laboratory experiments have been conducted to evaluate the effectiveness of several of these transgenic lines of cotton for control of Lepidopterous insects (19, 31-37). Cotton is a crop in the U.S. that does not cross pollinate with any wild or cultivated plants other than cotton and no wild cottons occurred where these experiments were conducted. Thus, there was no chance for escape of the genetically engineered gene to other species via pollen. A biological sink (buffer) of 24 rows of non-transgenic cotton was grown on all sides of the plots. This provided a place for the insects to deposit pollen when they visited flowers of the transgenic plants. Seed cotton from the 24 rows of cotton were destroyed. In an experiment in Mississippi to determine the efficacy of the 24 row border as a pollen sink, we found a significant reduction in pollen dissemination as distance from the test plot increased. Outcrossing went from 5% to < 1% by 7 m away from the test plot. A low level of pollen dispersal of < 1% continued to occur sporadically in the remaining border rows out to a distance of 25 m. The border rows fulfilled their purpose of serving as a pollen sink to significantly reduce the amount of pollen dissemination from the test plot (38). Cotton is normally self pollinated and cross pollinated only by insects. Wind is not a factor in cross pollination as the cotton pollen is heavy and not wind-borne. These features provided for a relatively safe experiment with transgenic cotton in field plots. For the two years of field tests that followed this experiment (38), regulatory agencies have accepted a 24 row border as sufficient pollen containment for the B. thuringiensis gene for field experiments with transgenic cotton.

A major economic factor in cotton production is the cost of insect control. Insects in the order Lepidoptera, which in the larval stage are worms or caterpillars, are major pests of cotton. In the mid-south about $123.00 US per ha, per year are spent on insect control. Most of this expense is for control of larva of Lepidoptera.

The gene from B. thuringiensis was genetically engineered into the Coker 312 cultivar of cotton via A. tumefaceins technology. The gene expressed in all cotton tissue and was measured in terminal leaves at a level of 0.05% to 0.10% of the total soluble protein (30). Six cotton lines, each with a different insertion of an insect control protein gene from B. thuringiensis, were evaluated in my laboratory (19). Each time a gene is engineered into cotton the placement of the gene among the cotton chromosomes is uncontrolled, but we do not know that it is random. Further, somaclonal variation and mutation can occur in tissue culture. Thus, each line is the result of a separate gene insertion and tissue culture processes. Because of this, each transformation event may be expressed differently. This may result in differences in the level of insect control protein and insect control. However, each transgenic line and the non-transformed Coker 312 should be considered as a pair of "near isogenic" lines for comparison purposes.

The field experiment conducted by my laboratory (19) involved 6 replications of 4 row plots each 9 m long separated by 1 m. The four rows were separated by 2 m blank rows. Plots were infested with 10-12 neonate tobacco budworm, Heliothis virescens, larvae once each week for 5 weeks beginning the second week of squaring. No insecticide was used for tobacco budworm control; however, malathion for boll weevil, Anthonomus grandis Boheman, and plant bug, Lygus spp., control was used. An identical set of plots were planted and not infested with tobacco budworm larvae. These plots were sprayed each week with a pyrethroid insecticide plus malathion to control all major insects. The difference in yield between the two sets of plots should reflect the amount of yield lost to the tobacco budworm insects and is an indirect measure of resistance to tobacco budworm provided by the genetically engineered insect control protein gene. A second measure of control provided by the B. thuringiensis gene was damaged squares or bolls caused by the weekly infestations of tobacco budworm.

Results of these field experiments show that the genetically engineered cotton lines were successful in controlling tobacco budworm insects and preventing excessive damage to the cotton plants (19). Transgenic lines never had over 8% damaged squares, whereas the non-transgenic strain had up to 22% damaged squares. Transgenic strains never had over 5 larvae per 100 squares compared to up to 19 larvae per 100 squares in Coker 312. The non-transgenic Coker 312 had up to 20% of the bolls damaged by tobacco budworm whereas boll damage ranged from 2 to 12% in the transgenic strains. Larvae in bolls ranged from 0 to 7.8 per 100 bolls in transgenic strains, but up to 22 per 100 bolls in the non-transgenic strains (19).

In the plots without pest insects, each of the transgenic strains yielded equal to or significantly more lint than Coker 312. This shows that the foreign gene in these cotton lines was not detrimental to yield. When grown under tobacco budworm infestation, each transgenic strain yielded significantly more than Coker 312. This shows that the B. thuringiensis gene in the transgenic plants was providing a significant and useful level of protection from damage by tobacco budworm (19).

Tobacco budworm larvae were grown in the laboratory on several plant parts each week beginning at seedling emergence and continuing until the end of the effective squaring period. The delta endotoxin was expressed in each plant part (39). When neonate tobacco budworm larvae were placed on anther and stigma tissue for 6 days the following results were obtained: Larval survival (mean of transgenics 1.7% ± 0.7:Coker 312 62.5% ± 7.5); Larval biomass (mean of transgenics 0.2 mg ± 0.2:Coker 312 117 mg ± 14.6) (39). Thus, the small amount of larval survival on the transgenics was not biologically significant because the larvae were very small and would not survive to pupation. Detailed data from the 1991 experiments cannot be cited due to confidentiality agreements between my laboratory and the company that provided the transgenic lines. However, the 1991 data fully support the above cited data, and the transgenic cotton lines provided effective field control of tobacco budworm.

Thus, we have shown that the gene from B. thuringiensis which encodes for a delta endotoxin can be genetically engineered into cotton and that the gene product (delta endotoxin) is expressed in a form that is effective in protecting transgenic cotton plants from damage by tobacco budworm. This represents an environmentally desirable way to control tobacco budworm in cotton.

Although the transgenic lines yielded as much or more than Coker 312 when the insects were controlled, we measured differences in some other traits. Bolls on 4 of the 6 transgenic lines were significantly smaller than Coker 312. Weight of 100 seed was less on two transgenic lines than on Coker 312 (19). In other research with five of these transgenic lines, each transgenic line had lower lint percentages and three had smaller bolls than Coker 312 (36). Effects on fiber properties have also been reported (33). At this time we cannot determine if these differences are due to pleitrophic effects of B. thuringiensis genes, position effects, or some (somaclonal) changes that occurred during tissue culture.

Other research has shown that these cotton lines also control cotton bollworm, Helicoverpa zea (32-35); pink bollworm, Pectinophora gossypiella; cotton leaf perforator, Bucculatrix thurberiella Busck; and saltmarsh caterpillar, Extigmene acrea (Drury) (36, 37); and tobacco budworm and cabbage looper, Tricoplusia ni (33); in field plots. These are the major Lepidopterous pests of cotton in the United States and are among the major worm pests of cotton worldwide.

Transgenic cotton with the gene from B. thuringiensis should be available to growers in the mid 1990's. These cotton cultivars will be marketed through the conventional channels of the existing cotton seed breeding companies. An Experimental Use Permit from the Environmental Protection Agency has been received for 1992 by at least one U. S. company involved in this research. Approval will be required from APHIS and/or EPA before the cotton cultivars can be placed into commercial production. Companies are presently collecting the research data to request granting of this status by the mid 1990's. Present indications are that cotton will be the first field crop to be grown commercially with a gene from B. thuringiensis that encodes for the delta endotoxin expressed at a level to provide effective control of several major Lepidopterous pests.

Acknowledgments

Transgenic cotton lines used in the experiments in Mississippi described under the heading "Results of Experiments with Bacillus thuringiensis Genes in Cotton" were developed by The Agricultural Group of Monsanto Company. This research was a cooperative effort between USDA, ARS, and Monsanto Company under Cooperative Research and Development Agreement No. 58-32U4-0-97.

Literature Cited

1. Khush, G. S.; Brar, D. S. Adv. Agron. 1991, 45, 223-274.
2. Hatchett, J. H.; Starks, K. J.; Webster, J. A. In Wheat and Wheat Improvement; Heyne, E. G., Ed.; ASA, CSSA, SSSA: Madison, WI, 1987; pp. 625-675.
3. Khush, G. S. Ann. NY Acad. Sci. 1977a, 228, 296-308.
4. Khush, G. S. Adv. Agron. 1977b, 29, 265-341.
5. Wharton T.; Jenkins, J. N.; McCarty, J. C., Jr.; Parrott, W. L. Crop Sci. 1991, 31, 29-32.
6. Fraley, R. T. Bio/Technology 1992, 10, 40-43.
7. Klein, T. M.; Arentzen, R.; Lewis, P. A.; Fitzpatrick-McElligott, S. Bio/Technology 1992, 10, 286-291.
8. Finer, J. J. Plant Cell Reports 1988, 7, 399-402.
9. Finer, J. J.; McMullen, M. D. Plant Cell Reports 1988, 8, 586-589.
10. Stewart, J. McD. CAB International, International Cotton Advisory Committee 1991, 49 pages.
11. Gasser, C. S.; Fraley, R. T. Science 1989, 244, 1293-1299.
12. della Chioppa, G.; Bauer, S. C.; Taylor, M. C.; Rochester, D. E.; Klein, B. K.; Shay, D. M.; Fraley, R. T.; Kishore, G. M. Bio/Technology, 1987, 5, 579-584.
13. Smith, C. J. S.; Watson, C. F.; Ray, J.; Bird, C. R.; Morris, P. C.; Schuch, W.; Grierson, D. Nature 1988, 334, 724-726.
14. Edwards, D. L.; Payne, J.; Soares, G. G. U. S. Patent 4,948,734. 1990.
15. Feitelson, J. S.; Payne, J.; Kim, L. Bio/Technology 1992, 10, 271-275.
16. Barton, K. A.; Whiteley, H. R.; Yang, Ning-Sun. Plant Physiol. 1987, 85, 1103-1109.
17. Umbeck, P.; Johnson, G.; Barton, K.; Swain, W. Bio/Technology 1987, 3, 263-266.
18. Jenkins, J. N.; Parrott, W. L.; McCarty, J. C.; Barton, K. A.; Umbeck, P. F. Miss. Agric. Forestry Expt. Stn. Bull. 1991, 174, 6 pp.
19. Jenkins, J. N.; Parrott, W. L.; McCarty, J. C. In Proceedings Beltwide Cotton Conferences; Herber, D. J., Ed.; National Cotton Council: Memphis, TN, 1991; p. 576.
20. Hilder, V. A.; Gatehouse, A. M. R.; Sheerman, S. E.; Barker, R. F.; Boulter, D. Nature 1987, 330, 160-163.
21. Gatehouse, A. M. R.; Gatehouse, J. A.; Boulter, D. Phytochemistry 1980, 751-756.
22. Hofte, H.; Whiteley, H. R. Microbiol. Rev 1989, 88, 242-255.
23. Perlak, F. J., Fuchs, R. L.; Dean, D. A.; McPherson, S. L.; Fischoff, D. A. Proc. Natl. Acad. Sci. (USA) 1991, 88, 3324-3328.
24. Stone, T. B.; Sims, S. R.; Marrone, P. G. J. Invertebr. Pathol. 1989, 53, 228-234.
25. McGaughey, W. H. Science 1985, 229, 193-195.
26. Van Rie, J.; McGaughey, W. H.; Johnson, D. E.; Barnett, B. D.; Van Mellaert, H. Science 1990, 247, 72-74.
27. Tabashnik, B. E.; Cushing, N. L.; Finson, N.; Johnson, M. W. J. Econ. Entomol. 1990, 83, 1671-1676.

28. Ferre, J.; Real, M. D.; Van Rie, J.; Jansens, S.; Peferoen, M. Proc. Natl. Acad. Sci. (USA) 1991, 88, 5119-5123.
29. Williams, S.; Friedrich, L.; Dincher, S.; Carozzi, N.; Kessmann, H.; Ward, E.; Ryals, J. Bio/Technology 1992, 10, 541-543.
30. Perlak, F. J.; Deaton, R. W.; Armstrong, T. A.; Fuchs, R. L.; Sims, S. R.; Greenplate, J. T.; Fischoff, D. A. Bio/Technology 1990, 8, 939-943.
31. Deaton, R. In Proceedings Beltwide Cotton Conferences; Herber, D. J., Ed.; National Cotton Council: Memphis, TN, 1991; p. 576.
32. Williamson, D. R.; Deaton, W. R. In Proceedings Beltwide Cotton Conferences; Herber, D. J., Ed.; National Cotton Council: Memphis, TN, 1991; p. 577.
33. Benedict, J. H.; Altman, D. W.; Sachs, E. S.; Deaton, W. R.; Ring, D. R. In Proceedings Beltwide Cotton Conferences; Herber, D. J., Ed.; National Cotton Council: Memphis, TN, 1991; p. 577.
34. Micinski, S.; Caldwell, W. D. In Proceedings Beltwide Cotton Conferences; Herber, D. J., Ed.; National Cotton Council: Memphis, TN, 1991; p. 578.
35. Gannaway, J. R.; Rummell, D. R.; Owen, D. F. In Proceedings Beltwide Cotton Conferences; Herber, D. J., Ed.; National Cotton Council: Memphis, TN, 1991; p. 578.
36. Wilson, D. F.; Flint, H. M.. In Proceedings Beltwide Cotton Conferences; Herber, D. J., Ed.; National Cotton Council: Memphis, TN, 1991; p. 579.
37. Wilson, F. D.; Flint, H. M.; Deaton, W. R.; Fischoff, D.; Armstrong, T. A.; Fuchs, R. L.; Berberich, S. A.; Parks, N. J.; Stapp, B. R. J. Econ. Entomol. 1992, In press.
38. Umbeck, P. F.; Barton, K. A.; Nordheim, E. V.; McCarty, J. C., Jr.; Parrott, W. L.; Jenkins, J. N. J. Econ. Entomol. 1991, 84, 1943-1950.
39. Jenkins, J. N.; Parrott, W. L.; McCarty, J. C.; Callahan, F. E.; Berberich, S. A.; Deaton, W. R. J. Econ. Entomol. 1992, In press.

RECEIVED July 31, 1992

Chapter 20

Recombinant Baculoviruses Expressing Foreign Genes for Insect Pest Control

Susumu Maeda[1] and Bruce D. Hammock[2]

**[1]Department of Entomology and [2]Department of Environmental Toxicology,
University of California, Davis, CA 95616**

A general overview of the use and safety of recombinant baculoviruses in insect control and their potential for commercial use in the near future is given in this chapter. A critical evaluation of recent advances in the use of recombinant DNA technology to enhance the insecticidal activity of naturally occurring insect viruses also is presented. This article targets scientists with a background in classical insecticide toxicology. Background references including recent advances in the molecular biology and use of baculoviruses essential in evaluating genetic modification of these viruses for insect control are presented.

Insect Viruses as Insecticides

Virally induced diseases of insects have historically been studied with regard to beneficial insects such as honey bees and silkworms. The use and study of insect viruses for pest control began in Canada in the 1930's with the introduction of a nuclear polyhedrosis virus (NPV) (a subfamily of Baculoviridae) for control of the European spruce sawfly, *Gilpinia hercyniae*. *G. hercyniae* NPV was used as an efficient insecticide to protect pine trees in North America (*1*) until replaced by synthetic chemical insecticides in 1960. Several species of insect viruses have also been introduced with varying success in the forests of Europe, North America, the former Soviet Union, and Japan. In the 1970's *Heliothis zea* NPV was made commercially available in the U.S. for the control of *H. zea* caterpillars which attack cotton, however, its use was limited predominantly due to its slow speed of insect kill and the introduction of faster acting synthetic chemical insecticides. The successful use of NPVs in industrialized countries for vegetable crop protection has been limited, however, in Brazil the *Anticarsia gammatalis* NPV has been used for the large scale protection of soybeans (*2*) and in the South Pacific the *Oryctes rhinoceros* baculovirus has been used for protection of palm and coconut trees (*3*). In addition to NPVs, cytoplasmic polyhedrosis viruses have also been used as effective insecticides (*4*). There are several other examples of the successful control of specific insects by insect viruses on a small scale. Compared to classical synthetic insecticides, however, their use has been very minor.

0097–6156/93/0524–0281$06.00/0

Insect viruses are classified into eight families: Poxviridae, Baculoviridae, Polydnaviridae, Iridoviridae, Parvoviridae, Reoviridae, Biruaviridae, and Picornaviridae (*5*). Baculoviridae is the most common family of insect viruses and more than 500 species of baculoviruses have been isolated (*6*). Baculoviruses generally have a limited spectrum of target insects, however, almost all major agricultural insect pests belonging to the order Lepidoptera are attacked by at least one species of baculovirus. Baculoviruses have been shown to be harmless to vertebrates and most beneficial arthropods (*7*). These and other attributes make baculoviruses excellent candidates for use in integrated pest management programs and under conditions where resistance to classical insecticides is a serious problem.

Classical synthetic insecticides have set a very high standard of efficacy, cost effectiveness and ease of use. Even in limited markets, baculoviruses will need to meet or exceed these standards before they can compete effectively with classical insecticides. The major disadvantage of naturally occurring baculoviruses in many cropping situations is their slow speed of kill. Recombinant DNA technology offers several routes to overcome this limitation of naturally occurring viruses for commercial exploitation. Refinements in the formulation and production of insect viruses for commercial use in crop protection are likely to be addressed along a number of fronts.

Replication and Gene Structure of Baculoviruses

Baculoviruses are characterized by genomes of closed circular double-stranded DNA. Viral particles of baculoviruses are rod shaped with an envelope size of 40-60 x 200-400 nm (*5*). Baculoviruses have been isolated from the insect orders Lepidoptera, Coleoptera, Diptera, Hymenoptera, Trichoptera, and from the crustacean order, Decapoda. No baculovirus counterparts have been found in plants, vertebrates, or invertebrates other than arthropods.

Baculoviruses are classified into three subfamilies: 1) nuclear polyhedrosis viruses (NPVs), 2) granulosis viruses (GVs), and 3) non-occluded baculoviruses. In the replication cycle of NPVs two types of viral progeny are formed: during an early stage of infection a budded form is released from the plasma membrane of infected cells; and during a late stage of infection an occluded form which is embedded in polyhedral-shaped inclusion bodies (Figure 1) is found in the nucleus (*8*). The budded form is necessary for cell to cell secondary infection in both *in vitro* cell culture and in host larvae. The occluded type is necessary for oral infectivity and prolonged protection of the virus in the field. Following ingestion of polyhedral inclusion bodies by host insect larvae, viral particles (occluded form) are released in the alkaline pH in the gut and attach to the midgut epithelial cells. After initial replication in midgut tissues, the viruses pass through the basal membrane of the midgut into the body cavity and start rapid replication in hemocytes and other tissues.

Baculoviruses as Expression Vectors

Baculoviruses have been successfully used as excellent eucaryotic expression vectors for basic and applied research (*9-11*). NPVs have several advantageous characteristics which make them ideal expression vectors including: 1) double stranded DNA genomes which can be modified easily, 2) established cell lines which support viral replication and allow the isolation and propagation of individual recombinant virus clones, 3) non essential genes, such as polyhedrin, which have strong promoters to drive foreign gene expression, and 4) markers for the detection of foreign gene insertion. Currently, *Autographa californica* NPV (AcNPV) and *Bombyx mori* NPV (BmNPV), are widely used as vectors for foreign gene

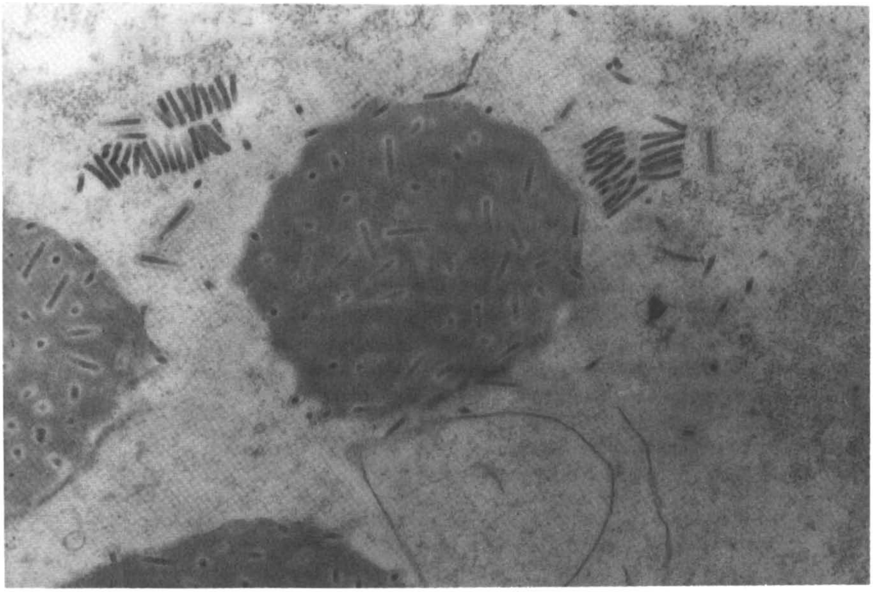


Figure 1. Electron microscopic observation of the nucleus of a fat body cell of the silkworm, *B. mori*, infected with BmNPV at a late stage of infection. Enveloped virions (occluded form) in the polyhedra and free nucleocapsids are visible.

expression in insect cells by replacement of the polyhedrin gene with a foreign gene. Although the construction and isolation of recombinant baculoviruses involves some technical skill and possibly more time compared to other expression systems (*12-13*), the benefits of high yield, ease of purification, correct post-translational modifications, authentic biological activity and antigenicity far outweigh any inconveniences (*9-11*). Recently several modified systems have become available through the open literature and in kit form which eliminate many of the difficulties and limitations of the baculovirus expression vector system as discussed below (*14-16*).

Only an overview of the construction of recombinant baculovirus for foreign gene expression is given in this chapter, since specific procedures for the construction of recombinant baculoviruses are described in detail in numerous publications (*12,13,17*). Due to the large size of the baculovirus genome (more than 100 kb), the recombinant virus must be constructed in two steps. Firstly, the foreign gene must be inserted into a transfer vector and propagated. Secondly, the foreign gene must be transferred from the transfer vector into the genome by homologous recombination. NPV transfer vectors generally contain an ampicillin gene and replication origin for propagation in *E. coli*, and a polylinker sequence for insertion of the foreign gene. Vectors are available with multiple cloning sites as well as with single cloning sites which fix the distance between the promoter and the transcriptional start site. The polylinker is flanked by the 5' and 3' (about 3 kb each) regions of the target gene (e.g. the polyhedrin gene) along with an appropriate promoter (e.g. the strong polyhedrin gene promoter).

Numerous transfer vectors with appropriate promoters are available for specific uses including: promoters from NPV genes other than the polyhedrin gene, e.g. p10 (*18*), chimeric promoters based on the polyhedrin gene promoter (*19*), and promoters derived from non-NPV genes, e.g. the hsp70 promoter from *Drosophila melanogaster* (*18*). Transfer vectors which simultaneously express two different foreign genes are available (*20*). Transfer vectors possessing the β-galactosidase gene (*16,18*) or complete polyhedrin gene (*14,21*) for ease of selection have greatly simplified cloning procedures. Transfer vectors possessing a functional polyhedrin gene can produce polyhedral inclusion bodies (occluded recombinant viruses) resulting in orally active viruses stable in the field.

Once the recombinant transfer vector is constructed, the foreign gene is transferred into the baculovirus genome by co-transfection with wild-type (or other appropriate) viral DNA in a susceptible cell line. After an appropriate incubation period recombinant viruses carrying the foreign gene are classically separated from wild-type (non-recombinant) viruses by plaque assay (generally by screening for the lack of polyhedral inclusion body production using a light microscope or by the naked eye for the absence of refractive polyhedra). Once isolated as a pure clone, the recombinant virus is propagated and the foreign gene is expressed in an established cell line or in larval insects. Either media containing proteins or serum free media can be used. Cells can be grown under a variety of conditions ranging from the surface of petri plates, through spinner flasks and roller bottles, to airlift fermenters and bioreactors. If necessary, the expressed product is purified by traditional biochemical procedures such as ion exchange column chromatography or affinity column systems.

The Use of Peptides for the Control of Insect Pests

A major public concern regarding synthetic chemical insecticides is their toxicity towards non-target animals. The appearance of resistant insect strains has also necessitated changes in traditional application methods and control strategies using chemical insecticides. Synthetic chemical insecticides are generally smaller than polypeptide molecules and induce toxic effects by binding to specific targets. Target sites such as those in the sodium channel of pest insects often have similar sites in vertebrate animals, including humans. The endocrine system presents novel targets for insect control. For example, the agonists and antagonists of two major insect hormones, juvenile hormone (JH) and ecdysone, have been considered and used successfully as insecticides (*22*) due to their ability to disrupt important insect metabolic functions. The neuroendocrine system relies on peptides as chemical mediators and has not been exploited for insect control. Also many toxins are peptidic. Peptides and polypeptides of insect and non-insect origin that can influence insect metabolism and metamorphosis can exhibit insecticidal effects without affecting other living organisms including vertebrates.

Polypeptide or Peptide Genes Useful for Insertion into the Baculovirus Genome

Baculoviruses are efficient vectors for carrying and expressing genes, possibly effective for insect pest control, into the insect body cavity. Numerous gene candidates have been considered for use in conjunction with baculovirus vectors for pest insect control. Any gene can be considered effective for pest insect control, if its expression can safely block feeding, disrupt metamorphosis, or otherwise change normal behavior so that there is a reduction in crop damage. It is not necessary for the product of the inserted gene to cause immediate insect death as long as there is a reduction in crop damage. Once the amino acid sequence of a peptide of interest has been determined, the nucleotide sequence of the gene which encodes the peptide can be deduced and constructed by chemical synthesis. Larger genes can be synthesized in parts and combined using plasmid vectors. Alternatively, cDNA or the authentic gene can be isolated from the organism producing the peptide of interest.

Candidate genes for construction of recombinant baculoviruses for insect pest control can be classified into the following categories:

Enzymes and Enzyme Inhibitors. Insect metabolism is controlled by numerous essential enzymatic pathways, many of which are tightly regulated in response to the environment. Various lethal mutants which are based on the inactivation or over-neuropeptide genes very attractive for construction of recombinant baculoviruses for pest control.

The amino acid sequences of several neuropeptides with potential for use in pest control have been determined. The amino acid sequence of an allatastatin (allatohibin) (*31*) and allatatropin (*32*) which inhibit and stimulate JH synthesis respectively, have been determined. The amino acid sequences of adipokinetic hormones (AKH), which stimulate the release of diglycerol from fat bodies, have been determined from various insects (*30*). AKH genes from *M. sexta* (*33*), and grasshoppers (*34*) have been isolated and characterized. Diuretic hormone, which plays an important role in the secretion of water, has also been isolated from *M. sexta* (*35*).

Genes encoding eclosion hormone, a signal for molting, from *M. sexta* (*36*) and *B. mori* (*37*) have been isolated and characterized. Prothoracicotropic hormone (PTTH) is a neuropeptide which targets the prothoracicotropic gland and initiates the synthesis and release of ecdysteroids. Genes encoding bombyxin (originally designated as 4 kD PTTH) and 22 kD PTTH, which exhibit PTTH activity to *Samia sincia* (*38*) and *B. mori* (*39*), respectively, have been isolated from *B. mori* and characterized.

Myotropin, leucopyrokinin, pheromone biosynthesis activating neuropeptide, and diapause hormone are neuropeptides which possess the consensus C-terminus sequence, Phe-Xaa-Pro-Arg-Leu-NH_2. Complete amino acid sequences of these peptides from several insects have been determined (*30,40*). These neuropeptides generally show unique biological activities, although cross biological activity also has been demonstrated (*41*, O. Yamashita, Nagoya University, personal communication, 1992).

Venom Toxins and Other Toxins. Venom is a toxin-containing fluid which is produced and stored in specialized tissues in venomous animals. Several species of venomous animals including scorpions, mites, spiders, and insect-predatory wasps paralyze their insect prey by injecting venom into them. Venoms from these animals generally contain neurotoxic components which are able to induce rapid paralysis at very low concentrations (as low as 10^{-12}M) (*42*). Many of these venoms are exceptionally specific for insects or a single insect group.

Scorpion Venom Toxins. The venoms of scorpions in the family Buthidae have various polypeptide neurotoxins that modify axonal sodium conductance. In 1971, a scorpion toxin which exhibits toxicity only towards insects, was isolated from the venom of the scorpion, *Androctonus australis* Hector (*43*). Insect selective scorpion toxins are classified into two groups: 1) excitatory insect toxins which cause immediate spastic paralysis, and 2) depressant insect toxins which induce progressive flaccid paralysis by blocking action potentials (*42*). Various electrophysiological and binding studies indicate that these two toxin groups exhibit toxicity only toward insects and show no cross reactivity to the mammalian specific alpha toxin (*42*).

The excitatory insect selective toxins, AaIT (AaIT1 and AaIT2) (*44,45*) and LqqIT1 (*46*), have been isolated from the scorpions, *A. australis* and *Leiurus quiniquestriatus quiniquestriatus*, respectively. AaIT injected into the body cavity of blowfly larvae causes immediate paralysis (*43*). Lepidopteran larvae, however, are about 500 times less sensitive to AaIT compared to the blowfly (*47*). AaIT is composed of 70 amino acid residues containing four disulfide bridges (Figure 2).

The depressant insect toxins LqqIT2 (*48*), LqhIT2 (*42*), and BjIT2 (*49*) have been isolated from the scorpions, *L. quiniquestriatus quiniquestriatus*, *L. quiniquestriatus hebraeus*, and *Burhorua judaicus*, respectively. These depressant insect toxins are composed of 61-64 amino acid residues and contain four disulfide bonds (*46*) (Figure 2).

LqhαIT, which exhibits high toxicity towards insects and very low toxicity towards mice, has been isolated from the scorpion *L. quiniquestriatus hebraeus* and characterized (*50*). This toxin binds to the same site bound by the mammalian specific alpha scorpion toxin.

Non-scorpion Venom Toxins. Insect-paralytic mites produce polypeptides which cause involuntary muscle contractions in lepidopteran insects. Two toxins, slow acting (250 kD) and immediate acting (around 21 kD), which cause paralysis in *Galleria mellonella*, have been isolated from the straw itch mite, *Pyemotes tritici* (*51*). Two closely related toxins, TxP-I (27 kD) and TxP-II (30 kD), have been further purified from immediate acting toxins. Both TxP-I and TxP-II are composed of about 8% cysteine. Both can cause muscle contraction and death upon injection into lepidopteran insect larvae (LD_{50}=500 ug/kg), however, they show no obvious effects (50 mg/kg) in mice (*52*). cDNA (Tox34) encoding TxP-I has been isolated using a specific antibody, and a synthetic oligomer probe based on the N-terminal amino acid sequence of TxP-I (*53*). The mode of action of the Tox34 gene product is not known, but preliminary experiments indicate that it acts presynaptically in a complex manner. It does not appear to have a simple effect on the sodium or calcium channels (L. K. Miller, University of Georgia, personal communication, 1992).

Certain spiders have toxins which can attack glutamate and calcium channel receptors. Several of these peptides, which are toxic only in insects, have been isolated and their amino acid sequences have been determined (*54*).

Insect-paralytic wasps are found in a wide variety of habitats and generally prey upon specific host insect species. Several of these wasps produce toxins which have potential for pest insect control. Wasps also often carry polydnaviruses in their calyx which are injected into host insects. Peptides expressed in the insect host cells by these polydnaviruses may cause paralysis of the host insects (*55*).

Bt Toxin. The bacterium, *Bacillus thuringiensis*, produces paracrystal insect specific endotoxins which have been widely used for pest control in many countries. Several types of Bt toxins which are toxic to Lepidoptera, Coleoptera, and Diptera, have been identified. Genes encoding these toxins have been isolated and the relationship between their host specificity and amino acid sequence have been examined. Many of the Bt toxins appear specific for midgut cells and require proteolytic activation, however, others appear to have cytolytic effects or act directly at the neuromuscular junction (*56,57*). The latter toxins or toxins acting on fundamental mechanisms of cell to cell communication are attractive for baculovirus expression.

Recombinant Baculoviruses Carrying Foreign Genes Causing Increased Insecticidal Effects for Insect Pest Control

Recombinant baculoviruses carrying various peptide genes have been constructed to see whether they exhibit any increased insecticidal effects. To date, the greatest increase in insecticidal activity reported has been obtained from recombinant baculoviruses carrying scorpion and mite toxin genes.

The BmNPV/*B. mori* expression system is an excellent system for screening genes with potential for pest insect control. The silkworm, *B. mori,* has been used for the screening of chemical insecticides for several decades at leading companies producing chemical insecticides in Japan. The silkworm is especially useful for

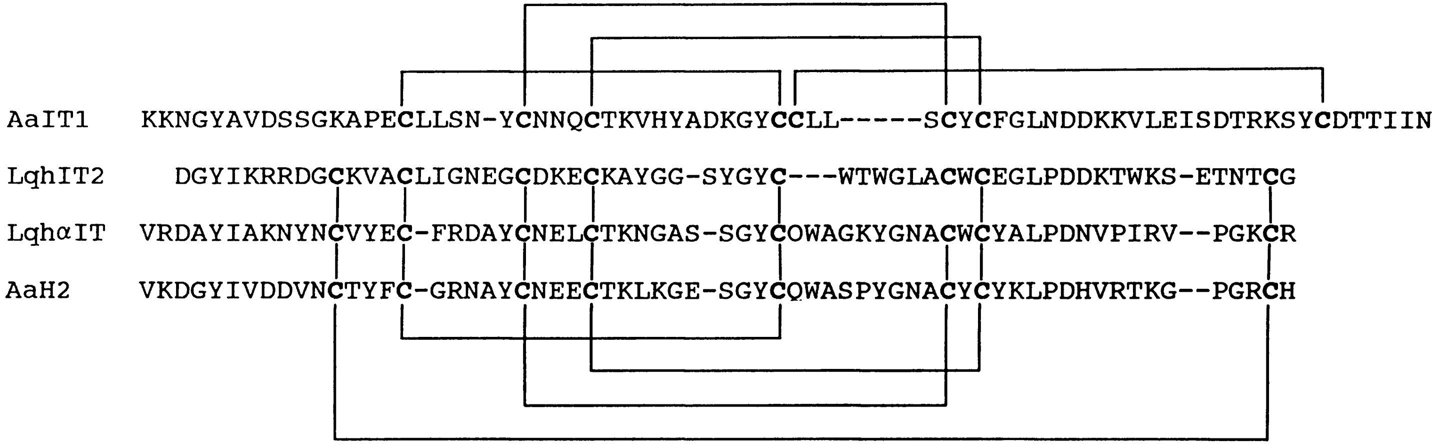

AaIT1 KKNGYAVDSSGKAPECLLSN-YCNNQCTKVHYADKGYCCLL-----SCYCFGLNDDKKVLEISDTRKSYCDTTIIN
LqhIT2 DGYIKRRDGCKVACLIGNEGCDKECKAYGG-SYGYC---WTWGLACWCEGLPDDKTWKS-ETNTCG
LqhαIT VRDAYIAKNYNCVYEC-FRDAYCNELCTKNGAS-SGYCOWAGKYGNACWCYALPDNVPIRV--PGKCR
AaH2 VKDGYIVDDVNCTYFC-GRNAYCNEECTKLKGE-SGYCQWASPYGNACYCYKLPDHVRTKG--PGRCH

Figure 2. Amino acid sequence comparison of scorpion toxins. AaIT1 and LqhIT2 are insect-specific scorpion toxins, LqhαIT shows high toxicity towards insects and low toxicity towards mammals, and AaH2 is mammalian-specific scorpion toxin (see *42-50*). Bars indicate the positions of disulfide bridges.

initial screenings due to the ease with which it can be synchronized and cultured at low cost and in large numbers. The response of *B. mori* to foreign peptides and chemicals is constant and comparable to other lepidopteran insects found in the wild. The silkworm, *B. mori*, is also very well characterized biochemically and physiologically. They have been domesticated for thousands of years and do not have the ability to survive in the field. This inability to survive in the field provides an added safety feature in regards to the use of this animal in recombinant DNA experiments by creating a major barrier to the escape of recombinant viruses. Following initial testing in the *B. mori* system, foreign genes are readily transferred to viruses infecting pest insects.

Recombinant baculoviruses carrying both a foreign gene for increased insecticidal activity and the polyhedrin gene for oral infectivity are the most feasible constructs for application of baculoviruses for crop protection in the field. The following polypeptides have shown increased insecticidal activity when expressed in lepidopteran larvae by a recombinant baculovirus.

Diuretic Hormone. A recombinant BmNPV carrying a diuretic hormone (DH) (*35*) gene under control of the strong polyhedrin promoter has been constructed (*58*). The complete DH gene cassette consisted of a secretion signal from a cuticle protein of *D. melanogaster*, the mature DH peptide gene, and a glycine codon for C-terminus amidation. The recombinant virus was constructed by replacing the polyhedrin gene in the BmNPV genome with the DH gene cassette by calcium mediated co-transfection. The recombinant BmNPV caused the hemolymph of infected *B. mori* larvae to excrete water into the midgut which resulted in about a 20-25% increase (about 24 h) in speed of insect kill compared to the wild-type virus.

JH Esterase. A recombinant AcNPV carrying cDNA encoding JH esterase of *H. virescens* under the control of the polyhedrin gene promoter (*28*) has been constructed. Injection of purified JH esterase expressed by the recombinant virus into the body cavity of *M. sexta* resulted in blackening, which is an indication of JH esterase induced reduction of JH titer in the hemolymph. JH esterase in infected larvae was detected specifically in pericardial cells by immunostaining (*59*). This was consistent with results showing a short half life and specific uptake of JH esterase injected into the body cavity of *M. sexta* (*60*, R. Ichinose, University of California, Davis, personal communication, 1992). Oral infection of first instar larvae of *T. ni* with high levels of the recombinant, polyhedron minus AcNPV inhibited their growth, however, this effect was not seen in other larval instars. Polyhedron positive constructs expressing JH esterase exhibited minimal effects in later larval instars regardless of the presence or absence of the ecdysone glucose transferase gene (*61-63*). Several modifications to JH esterase have been prepared by site directed mutagenesis and expressed under the p10 promoter. Two of these show enhanced speed of kill. One mutant acts slightly faster than the virus expressing AaHIT and one slightly slower (B. Bonning and V. Ward, University of California, Davis, personal communication, 1992).

Bt Toxin. Recombinant AcNPVs carrying the δ-endotoxin gene of *B. thuringiensis* subp. *kurstaki* HD-73 have been constructed (*64*). Recombinant AcNPV carrying the Bt toxin gene under control of the polyhedrin gene promoter produced toxin polypeptides of 130, 62, and 44 kD in *Spodoptera frugiperda* (Sf) cells, each of which were immunoreactive to antibodies specific to the Bt protoxin. Application of purified viral particles and cellular extracts from infected Sf cells to *T. ni* larvae exhibited inhibition of feeding and insect death which was presumably caused by the expressed toxin. In another recombinant AcNPV construct the same toxin gene was placed under control of the p10 promoter. This recombinant AcNPV produced occluded virus as well as biologically active Bt toxin. The LD_{50} of this polyhedron positive recombinant AcNPV was about two-fold higher and the LT_{50} was nearly the same as wild-type AcNPV. Bt toxin expression also reduced the expression of the late genes, p10 and polyhedrin, by 6-8 fold. This phenomenon may have been the result of cytotoxicity caused by the expressed Bt toxin.

Scorpion Toxins. A recombinant BmNPV carrying a synthetic toxin gene based on the *A. australis* scorpion toxin (AaIT) has been constructed (*65*). The inserted toxin gene consisted of a signal sequence for secretion from the bombyxin gene (*38*) and the coding sequence for synthetic AaIT. Injection of the recombinant BmNPV carrying the AaIT gene after the polyhedrin promoter into the body cavity of the silkworm, *B. mori*, induced symptoms expected from AaIT toxicity starting 40 hours post injection. Infected larvae exhibited characteristics including dorsal arching, body tremors, and feeding cessation which were identical to those observed following injection of authentic purified AaIT. The correct cleavage of the heterologous (bombyxin) signal sequence and secretion to the hemolymph was confirmed by N-terminal sequencing and SDS-PAGE analysis of the expressed AaIT. Compared to the wild-type virus a 40% increase in speed of insect kill was observed following injection of this recombinant virus (*65*). An increase in the speed of insect kill was not clearly observed in larvae infected with a similar recombinant BmNPV possessing the synthetic AaIT gene, but lacking the signal sequence for secretion. Larvae injected with authentic AaIT at low dosages recovered from the toxin induced symptoms. This indicates that accumulation of the expressed and secreted AaIT in the hemolymph is necessary for a decrease in time to insect death.

Two recombinant AcNPVs carrying the synthetic AaIT gene under control of the p10 promoter (the polyhedrin gene is active) have also been constructed (*66,67*). The toxin gene cassette was inserted into a polyhedrin gene positive transfer vector (*14*) and co-transfected with viral DNA from an AcNPV mutant with a deletion in the polyhedrin gene. The expression of AaIT was confirmed in orally-infected *H. virescens* (*67*) and *T. ni* (*66*) larvae using specific antibodies against AaIT and by bioassay. These viruses caused about a 30% increase in speed of insect kill and a clear reduction in feeding damage of cabbage leaves. The stability of these recombinant AcNPVs in the field should be similar to the wild-type virus due to their polyhedron positive phenotype.

Mite Toxin. A recombinant AcNPV carrying the mite toxin gene, Tox34, has been constructed using a transfer vector possessing a modified polyhedrin promoter, LSXIV (53,*68*). This virus excreted active toxin, which induced paralysis in a manner identical to that caused by authentic purified mite toxin, into the supernatant of infected Sf cells. Intrahemocoelic injection of this recombinant AcNPV caused paralysis in *G. mellonera* larvae within two days of infection. A similar recombinant AcNPV construct carrying a synthetic late gene promoter and a functional polyhedrin gene (*21*) also caused muscle contractions and exhibited an increase in speed of insect kill compared to wild-type AcNPV in *T. ni* larvae.

Improving Existing Viruses

The improvements in the biological activity of the baculoviruses expressing insect specific toxins and metabolic enzymes such as AaIT, TxP-1, and the modified JH esterases are significant in that they demonstrate the long held concept that the speed of kill can be dramatically improved by the expression of proteins deleterious to the insect. As discussed previously and elsewhere (*29*) the biological activity of these viruses can be improved by a variety of methods, however, a point of diminishing returns will eventually be reached with any of these approaches. It should also be noted that the research funds invested to date on improving the baculovirus' biological activity by all of the laboratories concerned are trivial in comparison to the funds invested in the development of similar leads using classical chemistry and second generation pesticide approaches (*69*).

Improvements in existing viruses can be based on the simple assumption that a lethal concentration in an active pool (for instance the hemolymph) must be reached before a desired effect is observed (death, cessation of feeding). As shown in Figure 3 by the heavy line I, insect death can be expected to occur when the concentration of protein exceeds the lethal concentration at 1. If this occurs before the normal time to death caused by the wild-type virus at 2, then a positive biological effect is seen. The same protein under a weaker or later promoter as shown by line II would not show an observable biological effect. Any of a variety of factors which result in an increased rate of production or decreased rate of removal/degradation from the active pool will decrease time to death as shown by line III resulting in death at 3.

In Figure 3, panel B, a hypothetical situation is shown where a toxin does not reach the lethal concentration rapidly enough to exhibit a biological effect as shown by line IV. If an increase in the rate of production and/or decrease in degradation can be accomplished as shown by line V, then an improved speed of kill is observed at time 4. The same effect can be obtained by placing the protein under an earlier promoter as shown by line VI. For instance Bonning et al. (*61,* personnel communication, 1992) report that both the basic protein promoter and the p10 promoter produce JH esterase earlier and at higher levels than the polyhedrin promoter. Tomalski and Miller, 1991 (*53*) also report higher expression of TxP-1 using a hybrid promoter. Further improvements will likely be seen as more information about promoter function becomes available and promoters from other organisms are isolated.

The rate of protein production is dependent upon numerous factors. Early in the infection process a relatively low number of cells will be infected and the concentration of the recombinant protein will be low even if a very strong early gene promoter is used (see Figure 3, panel C). If the same protein is expressed under both a strong late promoter (line I) and a weak early promoter (line VII), biological effects will only be observed with the strong late promoter because the protein never reaches the critical concentration under the early promoter. However, if removal/degradation of the protein can be halted, lethal concentrations can be reached even with the weak early promoter as shown by line VIII leading to death at 5. Alternatively if a highly potent toxin is used so that the lethal concentration is dramatically reduced from lethal concentration 1 to lethal concentration 2 (Figure 3, panel C) time of death can be reduced as indicated at 6.

It is very significant that the studies published to date move us from the region of low potency with no biological activity to a region where biological effects are observed. Toxins with greater potency will allow greater flexibility in using earlier and weaker promoters as shown in Figure 3, panel C. The effects of the peptides expressed by recombinant baculoviruses to date are of mediocre potency compared to others described (if not purified) in the literature. As increasingly more powerful factors are used, biological activity will be improved. A point of diminishing returns will be reached here as well, based upon the maximum rate of viral replication or other factors (Figure 3, panel D).

As shown in Figure 3, panels A, B, and C, the concentration of the deleterious protein or peptide must reach a critical concentration and be accessible to the active site. Three rate constants describe the removal of the toxin from the active pool: sequestration remote from the active site(s), metabolism, and excretion. A

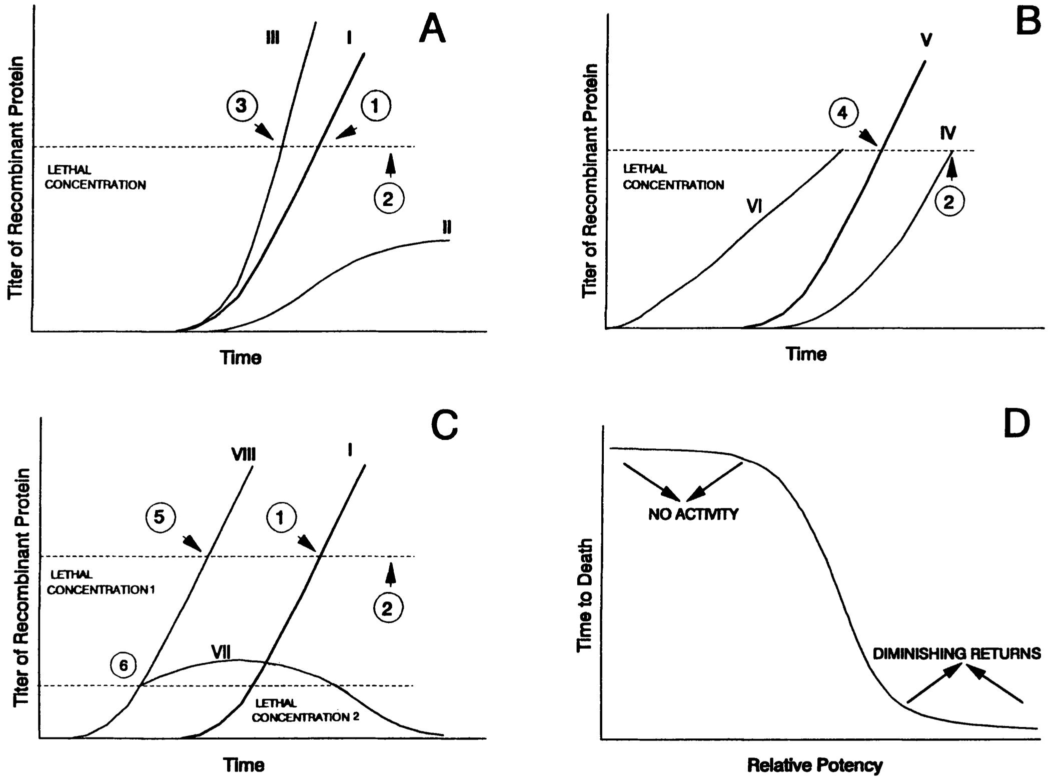


Figure 3. Panels A-C represent the effect of various factors as indicated in the text on the lethal concentration of recombinant protein. Panel D represents the effect of increasing the relative potency of a recombinant baculovirus in decreasing time to death.

higher concentration of protein in the active pool can be obtained by modifying the protein to decrease any of these rates. It is important to remember, however, that until these pathways of removal are saturated it can be anticipated that any increase in protein concentration in the active pool will result in a proportional increase in the amount of protein removed. Thus a series of sequential modifications in the toxin may be critical to increase the protein concentration in the active pool. In the simplest case this active pool is the hemolymph. However, another pool in more intimate contact with the active site(s) may be involved. In this case targeting of the viral infection or transport of the recombinant toxin could be critical.

The existing recombinant viruses if properly formulated can halve the time to death of infected insects. Since this translates to a great reduction in the amount of food consumed, this is an important advance for control of foliage feeders. How much more reduction is needed will vary with the crop concerned. Certainly even small improvements in time to kill will result in proportionally larger improvements in reduction of crop damage. For pests which attack fruiting parts of the plants, still greater speed of kill will be required. Using Figure 3 for planning, a variety of approaches can be used to improve the activity of recombinant baculoviruses. If these improvements in the recombinant viruses can be coupled with formulations resulting in better coverage or even attraction of the pest insects to virus droplets, effective control can be obtained even of larvae which bore quickly into the plant tissue. Times to death of under 24-48 h are likely to be reached with improvements in transcription, translation, export, targeting, and utilization of proteins of greater potency.

Safety and the Future

Two general approaches can be used to generate data regarding the safety of any pesticide, including recombinant organisms. One approach involves massive screening tests as currently done for classical compounds. The other approach involves the design of careful mechanistic tests which probe the safety of the organism (in this case the baculovirus), the expressed protein (mite toxin, scorpion toxin, JH esterase, etc.), and the foreign gene in its new environment (the recombinant organism). Certainly some general toxicity testing needs to be performed. However, incisive experiments designed to evaluate the safety of the recombinant organism are likely to be far more valuable and cost effective.

Like the wild-type baculoviruses, the recombinant organisms have many layers of selectivity. Baculoviruses embedded in the protein matrix of the inclusion bodies must be released under stringent conditions which dissolve the protein matrix without inactivating the virus particle. Non-occluded baculoviruses on the other hand are not generally orally infectious and are rapidly degraded in the field. Recombinant NPVs mixed with the wild-type virus become dramatically replaced with the wild-type virus during passages *in vitro* (*70*) indicating that the recombinant virus should be rapidly out competed by the wild-type virus in the field. Even if the virus is injected, most non-target insects will not be infected. Furthermore, recent studies have shown that baculoviruses are unable to enter into the mammalian cell nucleus (*71*). Thus, non-specific toxicity problems are very unlikely and if they do occur will most likely be observed on non-target lepidopteran larvae.

The safety of the use of baculoviruses in agriculture for pest control has been examined extensively and all studies unanimously indicate a high level of safety of baculoviral based insecticides (*7*). The viruses introduced in this chapter are designed as biological pesticides, not as classical biological control agents which will remain in the environment. The organisms are designed to not compete with the wild-type virus and will be rapidly eliminated in the field (*29*). Thus, it is unlikely that an organism will encounter the recombinant viruses unless that organism is in a specific microecosystem treated with the biological pesticide.

In addition to the selectivity of the baculovirus vector, the toxins expressed by the recombinant baculoviruses to date are highly insect selective. Although the toxins are peptides, they are expected to vanish rapidly from the environment. The toxin molecules are also not generally orally active (except in insects known to take up large proteins through the gut) even in species where they are active following infection with recombinant viruses.

The selectivity of scorpion toxin molecules has been studied extensively (*42*). The selectivity of AaIT in particular has been tested under very rigorous conditions including injection directly into the brains of mice with no detectable effects. It has also been demonstrated that AaIT fails to bind to the sodium channel of mammals. As indicated in Figure 2, AaIT only has about 30% amino acid homology to the mammalian specific α scorpion toxin, possesses an extended C-terminal amino acid chain and the positioning of its disulfide bridges are different (*72,42*). On the contrary, LqhIT2 (depressant insect toxin), LqhαIT (insect α toxin), and the mammalian specific α toxin have identical positioning of their disulfide bridges and they have about 40% and 70% amino acid homology, respectively, to the mammalian-specific toxin (*50,46,42*) (Figure 2). LqhαIT also has an identical binding domain in the target sodium channel as the mammalian specific α toxin and exhibits slowing and incomplete inactivation of sodium currents. LqhIT2, however, is insect specific and has a completely different binding domain compared to the α toxin (*48*) and LqhαIT exhibits very low toxicity in mice (*50*). These results indicate that the mechanisms of scorpion toxin selectivity are very restrictive and that AaIT appears to be a very safe molecule in terms of mammalian selectivity. Clearly other tests need to be done, but the data to date support the hypothesis that these recombinant viruses offer a large factor of safety.

Recombinant viruses carrying scorpion toxin genes, mite toxin genes, and modified JH esterases, have shown the greatest increase to date in speed of insect kill compared to wild-type virus and have exhibited effects similar to those caused by chemical insecticides. Modifications of the gene sequences of these polypeptides or other polypeptides effective for pest control or the use of stronger promoters or promoters activated during different stages of infection will increase the baculovirus natural insecticidal abilities. A combination of these approaches or the use of more potent/toxic gene products will increase the insecticidal ability of recombinant baculoviruses to levels comparable to those of chemical insecticides, however as with all technologies a point of diminishing returns will be reached in the ability of recombinant baculoviruses to control pest insects.

Acknowledgements

The assistance of Dr. Elizabeth Fowler (ABRU, Ciba Geigy Corp., Research Triangle Park, North Carolina) is gratefully acknowledged. Preparation of this manuscript was supported in part by the National Science Foundation (DCB-8818875), the U.S. Department of Agriculture (88-37234-4000, 91-37302-6186), National Institute of Environmental Health Sciences (ESO2710), and the North Atlantic Treaty Organization. B. D. Hammock is a Burroughs Wellcome Scholar in Toxicology.

Literature Cited

(*1*) Bird, F.T.; Burk, J.M. *Canad. Entomol.* **1961**, *93*, 228-238.
(*2*) Moscardi, F. *Mem. Inst. Osw. Cruz, Rie de Janeiro* **1989**, *84*, 51-56.
(*3*) Bedford, G.O. *Agric. Exosystems Environ.* **1986**, *15*, 141-147.

(*4*) Bird, F.T. *J. Insect Pathol.* **1961**, *3*, 352-380.
(*5*) Francki, R.I.B.; Fauquest, C.M.; Knudson, D.L.; Brown, F. *Classification and Nomenclature of Viruses*; Springer-Verlag: Wien, 1991; p 450.
(*6*) Martignoni, M.E.; Iwai, P.J. *General Technical Report PNW-159*; 4th Edition (Revised), USDA, 1986, 50 pp.
(*7*) Summers, M.; Engler, R.; Falcon, L.A.; Vail, P. *Baculoviruses for Insect Pest Control: Safety Considerations*, Amer. Soc. Microbiol., Washington D.C., 1975, 186 pp.
(*8*) Rohrmann, G.F. *J. Gen. Virol.* **1992**, *73*, 749-761.
(*9*) Miller, L.K. *Annu. Rev. Microbiol.* **1988**, *42*, 177-199.
(*10*) Maeda, S. *Annu. Rev. Entomol.* **1989**, *34*, 351-372.
(*11*) Luckow, V.A. In *Recombinant DNA Technology and Application*; Prokop, A.; Bajpai, R.K.; Ho, C.S., Eds.; McGraw-Hill, Inc., New York, NY, 1991, pp. 97-152.
(*12*) Summers, M.D.; Smith, G.E. *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures.* Texas Agric. Exper. St. Bull. No. 1555, 1987, 56 pp.
(*13*) Maeda, S. In *Invertebrate Cell System Applications;* Mitsuhashi, J., Ed.; CRC Press: Boca Raton, FL, 1989, Vol. I; pp. 167-181.
(*14*) Weyer, U.; Knight, S.; Possee, R.D. *J. Gen. Virol.* **1990**, *71*, 1525-1534.
(*15*) Kitts, P,A.; Ayres, M.D.; Possee, R.D. *Nucl. Acids Res.* **1990**, *18*, 5667-72.
(16) Vialard, J.; Lalumiere, M.; Vernet, T.; Briedis, D.; Alkhatib, G.; Henning, D.; Levin, D.; Richardson, C. *J. Virol.* **1991**, *64*, 37-50.
(*17*) O'Reilly, D.R.; Miller, L.K.; Luckow, V.A. *Baculovirus Expression Vectors: A Laboratory Manual.* W. H. Freeman and Company, New York, NY, 1992; 347 pp.
(*18*) Vlak, J.M.; Schouten, A.; Usmany, M.; Belsham, G.J.; Klinge-Roode, E.C.; Maule, A.; VanLent, J.W.M.; Zuidema, D. *Virology* **1991**, *179*, 312-320.
(*19*) Thiem, S.M.; Miller, L.K. *Gene* **1990**, *91*, 87-94.
(*20*) Weyer, U.; Possee, R.D. *J. Gen. Virol.* **1991**, *72*, 2976-2974.
(*21*) Xang, X.; Ooi, B.G.; Miller, L.K. *Gene* **1991**, *100*, 131-137.
(*22*) Staal, G.B. *Annu. Rev. Entomol.* **1986**, *31,* 391-429.
(*23*) Iwamura, H; Fujita, T. In *Pesticide Science and Biotechnology*; Greenhalgh, R.; Roberts, T.R., Eds.; Blackwell Scientific Publications: Oxford, UK, 1987, pp. 61-64.
(*24*) Wing, K.D.; Slawecki, R.A.; Carlson, G.R. *Science* **1988**, *241*, 470-472.
(*25*) Lerro, K.A.; Prestwich, G.D. *J. Biol. Chem. 265*, 19800-19806.
(*26*) Palli, S.R.; Osir, E.O.; Eng, W.S.; Boehm, M.F.; Edwards, M.; Kulcsar, P.; Ujvary, I.; Hiruma, K.; Prestwich, G.E.; Riddiford, L.M. *Proc. Natl. Acad. Sci. USA* **1990**, *87*, 796-800.
(*27*) Hammock, B.D. In *Comparative Insect Physiology Biochemistry and Pharamacology*; Kerkut, G.A.; Gilbert, L.I., Eds.; Pregamon Press: New York, NY, 1985, Vol. 7; pp. 431-472.
(*28*) Hammock, B.D.; Bonning, B.C.; Possee, R.D.; Hanzlik, T.N.; Maeda, S. *Nature* **1990**, *344*, 458-461.
(*29*) Hammock, B.D.; McCutchen, B.F.; Beetham, J.; Choudary, P.; Fowler, E.; Ichinose, R.; Ward, V.K.; Vickers, J.; Bonning, B.C.; Harshman, L.G.; Grant, D.; Uematsu, T.; Maeda, S. *Arch. Insect Biochem. Physiol.*, in press.
(*30*) Holman, G.M.; Nachman, R.J.; Wright, M.S. *Annu. Rev. Entomol.* **1990**, *35*, 201-217.
(*31*) Kramer, S.J.; Toshi, A.; Miller, C.A.; Kataoka, H.; Quistad, G.B.; Li, J.P.; Carney, R.L.; Schooley, D.A. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 9458-9462.
(*32*) Kataoka, H.; Troetschler, R.G.; Li, J.P.; Kramer, S.J.; Carney, R.L.; Schooley, D.A. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 2976-2980.

(*33*) Bradfield, J.Y.; Keeley, L.L. *J. Biol. Chem.* **1989**, *264*, 12791-12793.

(*34*) Noyes, B.E.; Schaffer, M.H. *J. Biol. Chem.* **1990**, *265*, 483-489.

(*35*) Kataoka, H.; Toshi, A.; Li, J.P.; Carney, R.L.; Schooley, D.A.; Kramer, S.J. *Science* **1989**, *243*, 1481-1483.

(*36*) Horodyski, F.M.; Riddiford, L.M.; Truman, J.W. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 8123-8127.

(*37*) Kamito, T.; Tanaka, H.; Sato, B.; Nagasawa, H.; Suzuki, Y. *Biochem. Biophys. Res. Comm.* **1992**, *182*, 514-519.

(*38*) Iwai, M.; Kawakami, A.; Ishizaki, H.; Takahashi, S.; Adachi, T.; Suzuki, Y.; Nagasawa, H.; Suzuki, A. *Develop. Growth Differ.* **1989**, *31*, 31-37.

(*39*) Kawakami, A.; Kataoka, H.; Oka, T.; Mizoguchi, A.; Kimura-Kawakami, Adachi, T.; Iwami, M.; Nagasawa, H.; Suzuki, A.; Ishizaki, H. *Science* **1990**, *247*, 1333-1335.

(*40*) Matsumoto, S.; Fonagy, A.; Kurihara, M.; Mitsui, T. *Biochem. Biophys. Res. Comm.* **1992**, *182*, 534-539.

(*41*) Imai, K.; Konno, T.; Nakazawa, Y.; Komiya, T.; Isome, M.; Koba, K.; Goto, T.; Yaginuma, T.; Sakakibara, K.; Hasegawa, K.; Yamashita, O. *J. Japan Academy, Series B* **1991**, *67*, 98-101.

(*42*) Zlotkin, E. *Phytoparasitica* **1991**, *19*, 177-182.

(43) Zlotkin, E.; Frankel, G.; Miranda, F.; Lissitzky, S. *Toxicon* **1971**, *9*, 1-8.

(*44*) Darbon, H.; Zlotkin, E.; Kopeyan, C.; van Rietschoten, J.; Rochat, H. *Int. J. Pept. Protein Res.* **1982**, *20*, 320-330.

(*45*) Loret, E.P.; Sampieri, F.; Roussel, A.; Granier, C.; Rochar, H. *Proteins: Structure, Function, and Genetics* **1990**, *8*, 164-172.

(*46*) Kopeyan, C.; Mansuelle, P.; Sampieri, F.; Brando,T.; Bahraoui, E.M.; Rochat, H.; Granier, C. *FEBS Lett.* **1990**, *261*, 423-426.

(*47*) Herrmann, R.; Fishman, L.; Zlotkin, E. *Insect Biochem.* **1990**, *20*, 625-637.

(*48*) Zlotkin, E.; Kadouri,;D.; Gordon, D.; Pelhate, M.; Martin, M.F.; Rocha, H. *Arch. Biochem. Biophys.* **1985**, *240*, 877-887.

(*49*) Lester, D.; Lazarovic, P.; Pelhate, M.; Zlotkin E. *Biochem. Biophys. Acta* **1982**, *701*, 310-387.

(*50*) Eitan, M.; Fowler, E.; Herrmann, R.; Duval, A.; Pelhate, M.; Zlotkin, E. *Biochemistry* **1990**, *29*, 5941-5945.

(*51*) Tomalski, M.D.; Bruce, W.A.; Travis, J.; Blum, M.S. *Toxicon* **1988**, *26*, 127-32.

(*52*) Tomalski, M.D.; Kutney, R.; Bruce, W.A.; Brown, M.R.; Blum, M.S.; Travis, J. *Toxicon* **1989**, *27*, 1151-1167.

(*53*) Tomalski, M.D.; Miller, L.K. *Nature* **1991**, *352*, 82-85.

(*54*) Skinner, W.S.; Adams, M.E.; Quistad, G.B.; Kataoka, H.; Cesarin, B.J.; Enderlin, F.E.; Schooley, D.A. *J. Biol. Chem.* **1989**, *264*, 2150-2195.

(*55*) Piek, T. *Venoms of the Hymenoptera: Biochemical, Pharmacological and Behavioural Aspects*; Academic Press: Orlando, FL, **1986**; pp 570.

(*56*) Cheung, P.Y.K.; Roe, R.M; Hammock, B.D.; Judson, C.L.; Montague, M.A. *Pest. Biochem. Physiol.* **1985**, *23*, 85-94.

(*57*) Sing, G.J.P.; Gill, S.S. *Pest. Biochem. Physiol.* **1985**, *24*, 408-414.

(*58*) Maeda, S. *Biochem. Biophys. Res. Commun.* **1989**, *165*, 1177-1183.

(*59*) Booth, T.F.; Bonning, B.C.; Hammock, B.D. *Tissue & Cell* **1992**, *24*, 267-282.

(*60*) Ichinose, R.; Kamita, G.S.; Maeda, S,; Hammock, B.D. *Pestic. Biochem. Physiol.* **1992**, *42*, 13-23.

(*61*) Bonning, B.C.; Hirst, M.; Possee, R.D.; Hammock, B.D. *Insect Biochem.*, in press.

(*62*) Roelvink, P.W.; van Meer, M.M.M.; de Kort, C.A.D.; Possee, R.D.; Hammock, B.D.; Vlak, J.M. *J. Gen. Virol.* **1992**, *73*, 1481-1489.

(*63*) Eldridge, R.; O'Reilly, D.R.; Hammock, B.D.; Miller, L.K. *Appl. Environ. Microbiol.* **1992**, *58*, 1583-1591.

(*64*) Merryweather, A.T.; Weyer, U.; Harris, M.P.G.; Hirst, M.; Booth, T.; Possee, R.D. *J. Gen. Virol.* **1990**, *71*, 1535-1544.

(*65*) Maeda, S.; Volrath, S.L.; Hanzlik, T.N.; Harper, S.A.; Majima, K.; Maddox, D.W.; Hammock, B.D.; Fowler, E. *Virology* **1991**, *184*, 777-780.

(*66*) Stewart, L.M.D.; Hirst, M.; Ferber, M.L.; Merryweather, A.T.; Cayley, P.J.; Possee, R.D. *Nature* **1991**, *352*, 85-88.

(*67*) McCutchen, B.F.; Choudary, P.V.; Crenshaw, R.; Maddox, D.; Kamita, S.G.; Palekar, N.; Volrath, S.L.; Fowler, E.; Hammock, B.D.; Maeda, S. *Bio/Technology* **1991**, *9*, 848-852.

(*68*) Ooi, B.G.; Rankin, C.; Miller, L.K. *J. Mol. Biol.* **1989**, 210, 721-736.

(*69*) Sparks, T.C.; Hammock, B.D. In *Pest Resistance to Pesticides: Challenges and Prospects*; Georghiou, G.P.; Satio, T., Eds.; Plenum Press: New York, NY, 1983, pp 615-668.

(*70*) Huang, Y.-S.; Bobseine, K.L.; Setzer, R.W.; Kawanishi, C.Y. *J. Gen. Virol.* **1991**, *72*, 2653-2660.

(71) Carbonell, L.F.; Miller, L.K. *Appl. Environ. Microbiol.* **1987**, *53*, 1412-1417.

(*72*) Gordon, D.; Jover, E.; Couraud, F.; Zlotkin, E. *Biochim. Biophys. Acta* **1984**, *778*, 349-358.

RECEIVED September 14, 1992

Chapter 21

Naturally Occurring Nematicides

David J. Chitwood

Nematology Laboratory, Beltsville Agricultural Research Center–West, Building 011A, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705

This review of naturally occurring phytochemicals with biological activity against plant-parasitic nematodes focuses on several diverse classes of compounds, including polythienyls, alkaloids, phenolics, polyacetylenes, fatty acids, terpenoids, and others. The nematotoxic mode of action and physiological role of most of these compounds in plants are unknown, although some are synthesized in response to nematode infection. Only a few plant families, primarily the Asteraceae and Fabaceae, have been examined for existence of nematotoxic substances. In a few cases, synthetic analogs of naturally occurring compounds have been synthesized and have possessed stronger biological activity than the naturally occurring ones. Enhanced research and development of phytochemical nematicides and analogs could provide safe, selective compounds for minimizing the multibillion dollar annual losses inflicted by phytoparasitic nematodes in the U.S.

Currently available chemicals for management of phytoparasitic nematodes are not only expensive but can also adversely affect the environment or human health (*1–3*). Consequently, several nematicides have been deregistered or restricted in use during the past decade, and others could face similar restrictions. Because phytoparasitic nematodes inflict annual agricultural losses of 6 billion dollars in the U.S. and 77 billion dollars in the world (*4*), better management tools are needed urgently.

Because higher plants could be expected to be a rich reservoir of interesting compounds with biological activity against phytoparasitic nematodes, isolation and identification of naturally occurring phytochemicals with biological activity against nematodes would be a logical first step for development of new, environmentally safe nematicides. Indeed, higher plants are being increasingly examined as sources for novel compounds with activity against animal-parasitic

**This chapter not subject to U.S. copyright
Published 1993 American Chemical Society**

nematodes (*5*). The development of agronomic nematicides is more difficult than development of mammalian anthelmintics because the former must either move rapidly within the soil without deactivation or else be systemic in plants (*6*). This review will focus on reports of compounds from higher plants with biological activity against phytoparasitic nematodes. Although direct toxicity is the most common biological activity of these compounds, several have other or unknown effects.

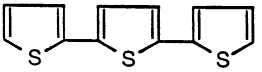
In keeping with the chemical orientation of the symposium, this review will not address studies demonstrating the activity of crude plant extracts against nematodes (*7–19*), although this research can lead to development of management strategies involving incorporation of plant extracts or residues into soil. Although the author's research involves the effects on nematodes of insect molting or juvenile hormones, compounds which do occur in the plant kingdom, this review will exclude these compounds because these investigations have been endocrinologically centered and have not involved plant-parasitic nematodes (*20–22*).

Polythienyls

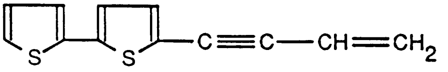
Marigolds (*Tagetes* spp., Asteraceae) were among the first plants to be examined for nematicidal compounds because they often suppress populations of soil nematodes. Two such compounds identified (Figure 1) were α-terthienyl (I) and 5-(3-buten-1-ynyl)-2,2'-bithienyl (II), with the former active against the stem and bulb nematode (*Ditylenchus dipsaci*) at 5 μg/ml, the wheat seed gall nematode (*Anguina tritici*) at 0.5 μg/ml, and the potato cyst nematode (*Globodera rostochiensis*) at 0.1–0.2 μg/ml (*23*, *24*). In a survey of 110 different Asteraceae, over 40 species suppressed greenhouse populations of the lesion nematode *Pratylenchus penetrans*, and thienyls occurred in at least 15 of these species. Two reviews (*25*, *26*) contain excellent summaries of the effects of marigolds on populations of nematodes in soils, the weak nematicidal activity of the two polythienyls or synthetic analogs when incorporated into soil, and the mode of action of the compounds, which involves generation of singlet oxygen by light, peroxidase, or other activators. Interestingly, a simple analog (tetrachlorothiophene) was once a registered nematicide in the U.S. but is now obsolete (*6*).

Alkaloids

Several naturally occurring alkaloids (Figure 2) are toxic or otherwise inhibitory to phytoparasitic nematodes. For example, physostigmine (III), an alkaloid from the Calabar bean, *Physostigma venenosum* (Fabaceae), immobilized *D.*



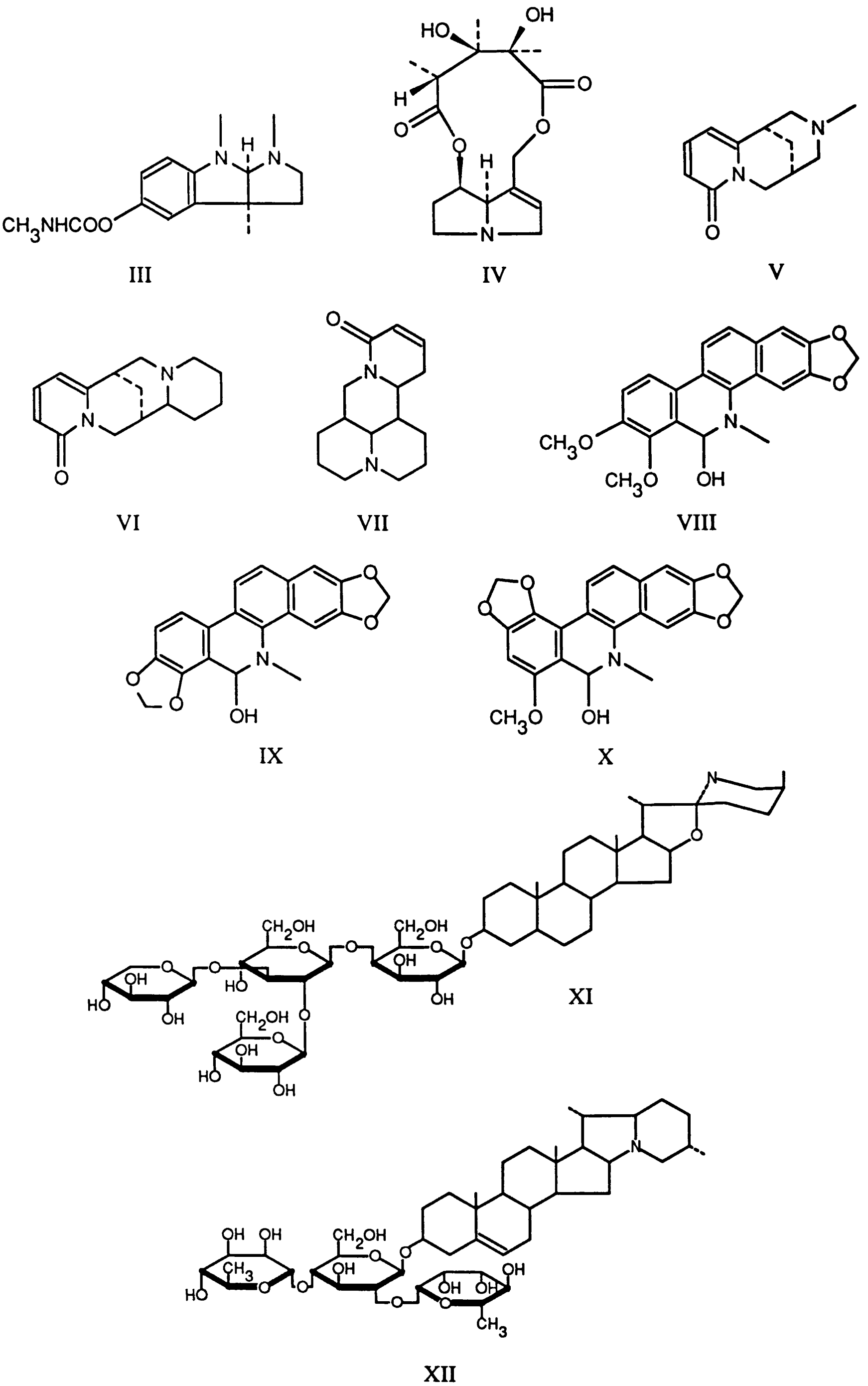
I



II

Figure 1. Polythienyls biologically active against nematodes.

In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.


CH3NHCOO
H
N
N
HO
OH
H
O
O
O
O
H
N
O
N
N
O
N
N
N
O
O
O
CH3O
CH3O
N
OH
O
O
N
O
O
OH
O
O
O
O
N
CH3O
OH
N
O
CH2OH
CH2OH
O
O
O
OH
OH
O
HO
HO
OH
OH
CH2OH
O
OH
HO
OH
N
OH
OH
CH2OH
CH3
O
OH
HO
O
O
O
OH
OH
OH
O
CH3

III IV V

VI VII VIII

IX X

XI

XII

Figure 2. Alkaloids biologically active against nematodes.

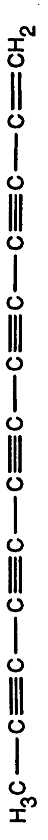
dipsaci at 1 mg/ml (*27*) but was not toxic, as nematodes regained motility and became infective after transfer to water. Pretreatment of pea seedlings with 30 μg/ml of physostigmine sulfate protected them against subsequent infection. Another bean alkaloid, monocrotaline (IV) (from the showy crotalaria, *Crotalaria spectabilis*) inhibited movement of juveniles of the root-knot nematode *Meloidogyne incognita* at 10 μg/ml (*28*). Resistance to *M. incognita,* however, was not correlated with the monocrotaline content of various species of *Crotalaria* or *Cynoglossum*. Three other alkaloids— *N*-methylcytisine (V), anagyrine (VI), and sophocarpine (VII)—were discovered in another member of the Fabaceae, *Sophora flavescens* (*29, 30*). When applied to cotton balls in petri dish cultures of the pinewood nematode (*Bursaphelenchus xylophilus*) feeding upon the fungus *Botrytis cinerea*, the alkaloids inhibited reproduction at 3–6 μg.

Three tetracyclic alkaloids—chelerythrine (VIII), sanguinarine (IX), and the previously unknown bocconine (X)—were identified in a poppy, *Bocconia cordata* (*31, 32*). Each was active at 50–100 μg/ml against the free-living nematodes *Rhabditis* and *Panagrolaimus*. The Solanaceous steroidal glycoalkaloid α-tomatine (XI) was also toxic to the free-living nematode *Panagrellus redivivus*; the ED_{50} expectedly varied with pH and was as low as 50 μg/ml (*33*). A similar pH-dependent response occurred with another Solanaceous alkaloid, α-chaconine (XII); the most effective ED_{50} was 85 μg/ml (*34*). A nonprotonated nitrogen atom was required for maximal activity of either compound.

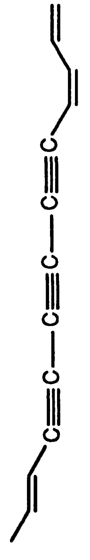
Acetylenes

Nematotoxic polyacetylenes (Figure 3) have been isolated from many members of the Asteraceae. The first of these was tridec-1-en-3,5,7,9,11-pentayne (XIII), isolated from *Helenium* sp. and active against *Pratylenchus penetrans* (*25, 35*). Subsequently, 3-*cis*, 11-*trans*- and 3-*trans*,11-*trans*-trideca-1,3,11-triene-5,7,9-triyne (XIV,XV) were obtained from flowers of safflower (*Carthamus tinctorius*) and were nematicidal against the rice white tip nematode (*Aphelenchoides besseyi*) at 1.0 μg/ml (*36, 37*). Two acetylenes, tridec-1-en-3,5,7,9,11-pentayne (XIII) and 9,10-epoxyheptadec-16-en-4,6-diyn-8-ol (XVI), were identified from roots of a thistle (*Cirsium japonicum*) and completely inhibited reproduction of *B. xylophilus* in the cotton ball bioassay at 16 or 250 μg, respectively (*38*). In the same bioassay, 1-phenylhepta-1,3,5-triyne (XVII) and 2-phenyl-5-(1'-propynyl)-thiophene (XVIII) from tickseed (*Coreopsis lanceolata*) and *cis*-dehydromatricaria ester (XIX) from goldenrod [*Solidago canadensis* (= *S. altissima*)] completely inhibited nematode reproduction at 110 μg/ball (*38*).

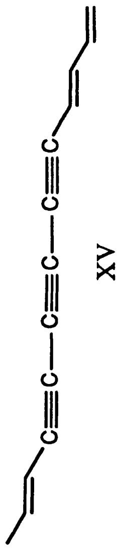
Roots of a daisy (*Erigeron philadelphicus*) contained methyl 2-*trans*,8-*cis*-deca-2,8-diene-4,6-diynoate (2-*trans*,8-*cis*-matricaria ester, XX) and methyl 2-*cis*,8-*cis*-deca-2,8-diene-4,6-diynoate (2-*cis*,8-*cis*-matricaria ester, XXI). The LD_{50} of each against the lesion nematode *Pratylenchus coffeae* was 3.0 μg/ml (*39*). Four additional naturally occurring polyacetylenes (*cis*- and *trans*-dehydromatricaria and *cis*- and *trans*-lachnophyllum esters, (XIX, XXII-XXIV) and four synthetic analogs (lachnophyllic acid, dehydromatricarianol, dehydromatricarianyl acetate, and lachnophyllol XXV-XXVIII) were also active. In an investigation of nematotoxicity against *P. coffeae* of 28 synthetic analogs,



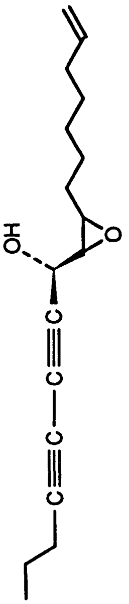
XIII



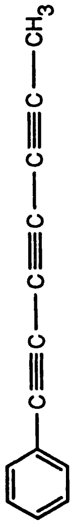
XIV



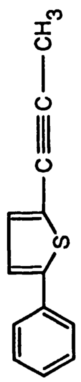
XV



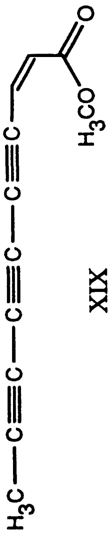
XVI



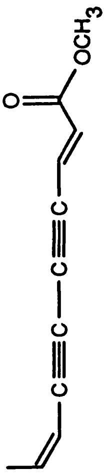
XVII



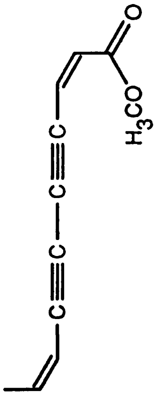
XVIII



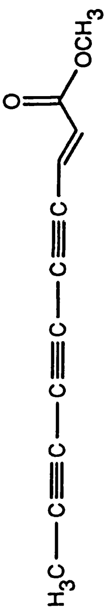
XIX



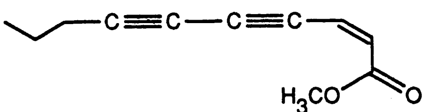
XX



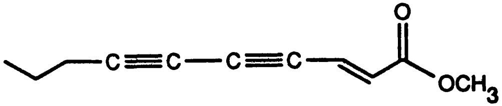
XXI



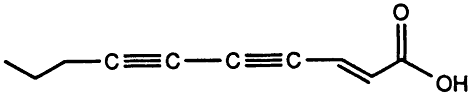
XXII



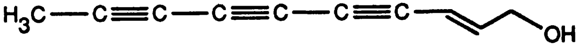
XXIII



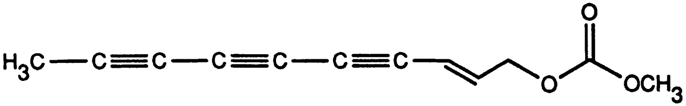
XXIV



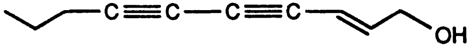
XXV



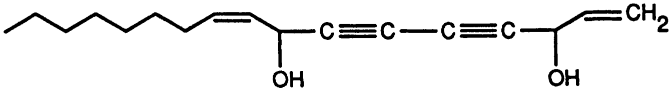
XXVI



XXVII



XXVIII



XXIX

Figure 3. Acetylenes biologically active against nematodes.

none with greater than one triple bond, several were active at concentrations below 1.0 µg/ml (*40*). A triple bond-conjugated ketone, aryl, or ester group resulted in maximal activity.

Finally, among non-Asteraceae, heptadeca-1,9-diene-4,6-diyne-3,8-diol (XXIX) from roots of *Angelica pubescens* (Apiaceae) had nematicidal activity against *Aphelenchoides besseyi* (*41*).

Fatty Acids and Other Lipids

Several lipids (Figure 4) are toxic to nematodes. At 1 mg/ml most of 41 fatty acids or their salts evaluated were toxic against *Panagrellus redivivus*, as were most of the 13 tested against the tobacco cyst nematode *Globodera tabacum* (*42*). Butyric acid (XXX) was identified in decomposing rye (*Secale cereale*) and timothy (*Phleum pratense*) and was toxic at 880 µg/ml to *M. incognita* and *P. penetrans* but not to the free-living nematodes *Rhabditis*, *Cephalobus*, and *Plectus* (*43*). Variation of activity with pH indicated that the undissociated acid was nematicidal. Extracts of *Iris japonica* (Iridaceae) roots were toxic to *A. besseyi*; purification by HPLC indicated that myristic, palmitic, and linoleic acids (XXXI–XXXIII) were nematicidal components (*41*). 2-Undecylenic acid was the most active (LD_{50} < 10 µg/ml) of the synthetic fatty acids tested.

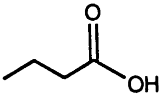
Di-*n*-butyl succinate (XXXIV) created artifactually during isolation and identification of nematicidal substances from peanut (*Arachis hypogaea*, Fabaceae) induced 90% mortality against *Pratylenchus coffeae* at 100 µg/ml (*44*). Eleven of 17 additional, synthetic dialkyl succinates were nematicidal.

Terpenoids

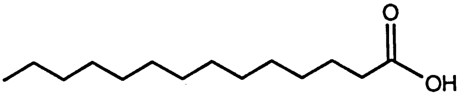
Several investigators have described nematotoxicity of various terpenoids (Figure 5). In a comparison of 20 nontraditional chemicals applied as root dips or soil drenches to tomato plants for control of the root-knot nematode *Meloidogyne javanica*, citral (XXXV) and geraniol (XXXVI) inhibited nematode reproduction by 52% and 86%, respectively, when applied as 100 µg/ml soil drenches (*45*). Similarly, when limonene (XXXVII), a component of citrus oil and an inhibitor of insect neurotransmission, was applied as a soil drench at 100 µg/ml, population development of the sugarbeet cyst nematode (*Heterodera schachtii*) was 3% of that of controls (*46*). Steam-distilled essential oils of several Labiatae and Myrtaceae were toxic to four species of plant-parasitic nematodes, as were five synthetic terpenoids identified in the extracts—geraniol (XXXVI), linalool (XXXVIII), eugenol (XXXIX), menthol (XL), and 1,8-cineole (XLI) (*47,48*). A foliar spray of eugenol decreased galling of okra (*Abelmoschus esculentus*) induced by *M. incognita* (*18*)

Phenolics

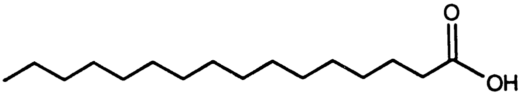
There has been substantial investigation of the role of elevated preinfectional levels of plant phenolics (Figure 6) in resistance to nematodes (*49, 50*).



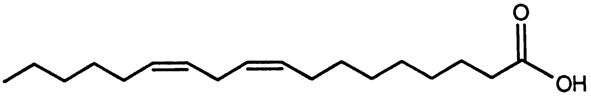
XXX



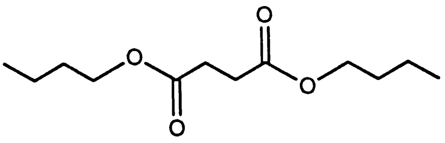
XXXI



XXXII



XXXIII



XXXIV

Figure 4. Fatty acids and other lipids biologically active against nematodes.

Unfortunately, few of these compounds have been isolated from plant roots and examined for nematotoxicity. Pyrocatechol (XLII), isolated from weeping lovegrass (*Eragrostis curvula*, Gramineae), was toxic to root-knot nematode juveniles (*51*). As a 400 µg/ml soil drench, the common plant phenolic quercetin (XLIII) inhibited reproduction of *M. javanica* (*45*).

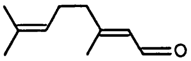
Postinfectional Compounds

Numerous compounds are synthesized by plants after nematode attack; a large body of work has accumulated on the role of these compounds (Figure 7) in resistance to nematodes (*25*, *26*, *49*, *50*, *52–57*). Although lack of space precludes a discussion of the function of postinfectionally synthesized compounds, several of these investigations are relevant to the scope of this article. For example, coumestrol (XLIV) was produced by lima beans in response to infection by *Pratylenchus scribneri* and inhibited the motility of *P. scribneri* at 5.0 µg/ml (*58*). Similarly, resistance to *M. incognita* in cotton (*Gossypium hirsutum*, Malvaceae) was associated with biosynthesis of terpenoid aldehydes; a crude terpenoid aldehyde extract from cotton inhibited movement of *M. incognita* at 50 µg/ml, as did gossypol (XLV) at 125 µg/ml (*59*, *60*). In soybean roots, glyceollin (XLVI) accumulated during incompatibility to root-knot nematodes. At 15 µg/ml, the compound strongly inhibited movement of *M. incognita* in vitro; nematodes recovered upon removal from glyceollin solutions (*61*, *62*). In potato tubers, levels of rishitin (XLVII) synthesized in response to infection by the potato rot nematode (*Ditylenchus destructor*) were correlated with resistance to nematodes, and the compound inhibited movement of *Ditylenchus dipsaci* at 100 µg/ml (*63*).

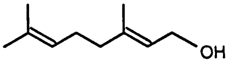
Other Compounds

Many additional compounds (Figure 8) possess antagonism towards plant-parasitic nematodes. The inhibition of hatching of eggs of *Globodera rostochiensis* by mustard seedlings was one of the first investigations of chemically mediated plant-nematode interactions. Allyl isothiocyanate (XLVIII), a constituent of seeds of black mustard (*Brassica nigra*, Cruciferae), inhibited hatching at 50 µg/ml and significantly improved potato yield when incorporated into field soils (*64*). Several isothiocyanates and related compounds have been evaluated as preplant soil fumigants, including sodium methyldithiocarbamate, a commercially utilized compound which degrades in soil to form methyl isothiocyanate (*2*).

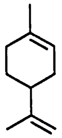
Resistance of garden asparagus (*Asparagus officinalis*, Liliaceae) to the stubby-root nematode (*Paratrichodorus christiei*) is associated with a preformed chemical substance in roots and root exudates; the compound was characterized as a glycoside with a low molecular weight aglycone (*65*). At concentrations of 100 µg/ml, the purified compound paralyzed four species of plant-parasitic nematodes, and root drenches or foliar sprays of tomato plants reduced populations of *M. incognita*. Unfortunately, the compound was not identified.



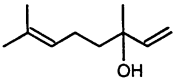
XXXV



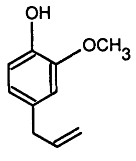
XXXVI



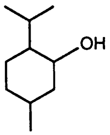
XXXVII



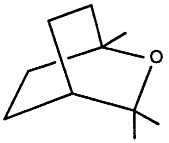
XXXVIII



XXXIX

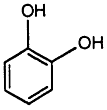


XL

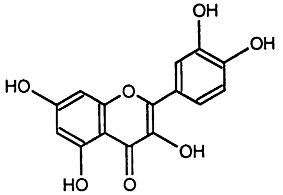


XLI

Figure 5. Terpenoids biologically active against nematodes.

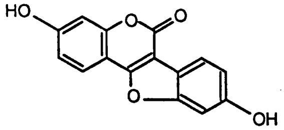


XLII

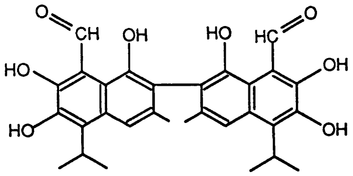

OH
OH
HO
O
OH
HO
O

XLIII

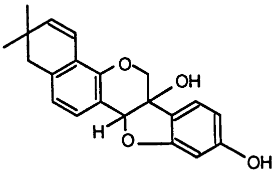
Figure 6. Phenolics biologically active against nematodes.



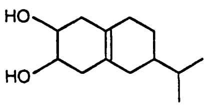
XLIV


O
CH
OH
HO
HC
O
HO
OH
HO
OH

XLV



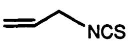
XLVI



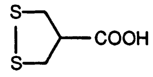
XLVII

Figure 7. Postinfectional compounds biologically active against nematodes.

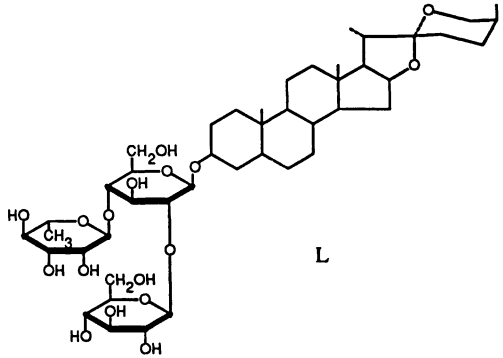
In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.



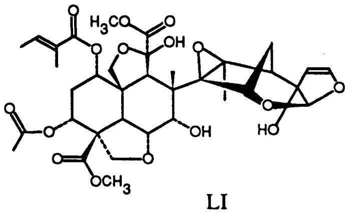
XLVIII



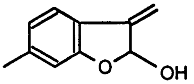
XLIX


CH2OH
OH
HO
CH3
OH
OH
CH2OH
OH
HO
OH
O

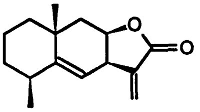
L


H3CO
O
OH
O
OH
HO
OCH3

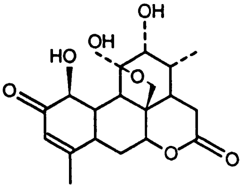
LI



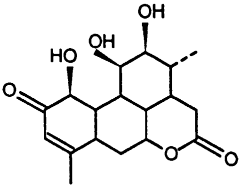
LII



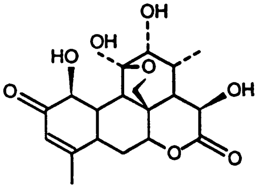
LIII


OH
OH
HO
O
O
O
O

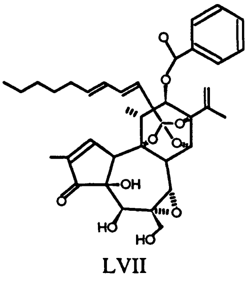
LIV



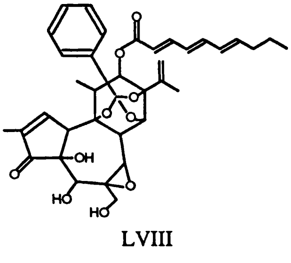
LV


OH
OH
HO
O
OH
O
O
O

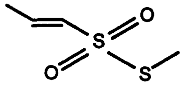
LVI



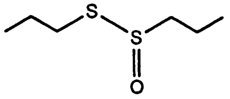
LVII



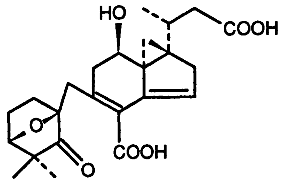
LVIII



LIX



LX


HO
COOH
O
COOH
O

LXI

Figure 8. Miscellaneous compounds biologically active against nematodes.

Much later, asparagusic acid (XLIX) was isolated from 30 kg of asparagus roots at a concentration of 35 μg/ml. It inhibited hatching of the soybean cyst nematode (*Heterodera glycines*) and *G. rostochiensis* at 50 μg/ml, even in the presence of hatching stimulants. The same concentration resulted in 80-99% mortality in *Meloidogyne hapla*, *Pratylenchus penetrans*, and the pin nematode *Paratylenchus curvitatus* (*66*). Another species of asparagus, *A. adescendens*, contained several glycosides, e.g., asparanin B (L), which inhibited motility of *M. javanica* at 200 μg/ml (*67*).

The nematicidal properties of the neem tree (*Azadirachta indica*, Meliaceae) have been reviewed (*68*). Although various plant tissues or crude extracts are nematicidal, the specific nematotoxic compounds are unknown. One neem component, azadirachtin (LI), is an insect antifeedant and growth and molt inhibitor (for references, see *69*). Because it inhibits microfilarial release in the animal-parasitic nematode *Brugia pahangi* at 10 μg/ml (*70*), azadirachtin is likely one of the many neem components active against plant-parasitic nematodes.

Other phytochemicals with nematicidal activity include 2,3-dihydro-2-hydroxy-3-methylene-6-methylbenzofuran (LII) from *Helenium* sp. (Asteraceae) (*71*) and alantolactone (LIII) from elecampane (*Inula helenium*, Asteraceae) (*72*). *Hannoa undulata* (Simarubaceae) seeds contained three polycyclic lactones: chaparrinone (LIV), klaineanone (LV), and glaucarubolone (LVI). A 1.0-μg/ml mixture of these compounds inhibited penetration of tomato roots by *M. javanica* (*73*). Roots of *Daphne odora* (Thymelaeaceae) contained odoracin (LVII) and odoratrin (LVIII), which induced 100% mortality in *Aphelenchoides besseyi* at ≤5.0 μg/ml (*41*, *74*). Four compounds were isolated from methanolic extracts of *Allium grayi* (Liliaceae) and were active in vitro against *M. incognita*: 1-octanol, methyl 4-hydroxybenzoate, methyl 4'-hydroxycinnamate, and allygrin (LIX) (*75*). *Allium fistulosum* var. *caespitosum* contained dipropyl thiosulfinate (LX) and four other thiosulfinates; each was more active than the four compounds from *A. grayi*. Many other thiosulfinates and thiosulfonates were prepared and had nematicidal and antibacterial activity (*75*).

Although hatching stimulants are not nematicidal, they could be used in field situations to induce hatching in the absence of host plants. Glycinoeclepin A (LXI), a hatching stimulant for *Heterodera glycines*, was isolated from roots of kidney bean (*Phaseolus vulgaris*, Fabaceae) (*76*); two related nortriterpenoids were also isolated but did not stimulate hatching (*77*). Two simpler analogs were synthesized chemically and stimulated hatching, but were less active biologically than glycinoeclepin A (*78*).

The involvement of plant lectins in plant-nematode recognition events is a very active area of research (*51*, *79*). Concanavalin A, a lectin from the jackbean, *Canavalia ensiformis* (Fabaceae), provided substantial control of *M. incognita* on tomato in growth chamber, greenhouse, and microplot experiments (*80*). Pretreatment of infective *M. incognita* juveniles with concanavalin A or two other lectins somewhat stimulated the hypersensitive response of soybean to the nematodes (*79*).

Future Prospects

The most conspicuous aspect of these investigations of nematicidal phytochemicals is that this field of research is truly in its infancy. Even though only a few plants have been investigated, a wide range of structurally diverse compounds with nematotoxic effects have been identified. Because only a few plant families (mainly the Asteraceae and Fabaceae) have been examined for antinematodal activity, a more extensive range of plant taxa needs to be investigated.

The functional roles of these compounds in plants have also been investigated to a limited extent. Although one speculation would be that such compounds function in higher plants as antinematodal substances, most researchers have noted that inadequate attention has been given in most cases to the concentrations of many of these compounds within plants, or their cellular or subcellular locations. Occasionally, the concentrations required for *in vitro* biological activity are sufficiently high to indicate that the compounds do not function *in vivo* as nematode antagonists.

From the perspective of an agricultural chemist, this field of research is in its infancy because most investigations have been of a basic, descriptive nature. In only a few cases have analogs of phytochemical nematicides been chemically synthesized and examined for biological activity. Similarly, except for the nematicidal components in marigolds, the antinematodal mode of action of these compounds has received slight attention. Of course, in some cases (e.g., polyacetylenes) there exists excellent studies of mode of action in other organisms. Regardless of mode of action, the high potential of naturally occurring compounds for environmental compatibility indicates that many phytochemicals could be valuable starting points in the development of safer management tools for phytoparasitic nematodes. The multibillion dollar losses caused by plant-parasitic nematodes and the problems facing current management practices dictates that research of natural products with activity against nematodes be intensified.

Acknowledgment. The author thanks Patricia Oakley for preparation of the figures.

Literature Cited

1. Hague, N. G. M.; Gowen, S. R. In *Principles and Practice of Nematode Control in Crops*; Brown, R. H.; Kerry, B. R., Eds.; Academic Press: Sydney, 1987, pp 131-178.
2. Johnson, A. W.; Feldmesser, J. In *Vistas on Nematology*; Veech, J. A.; Dickson, D. W., Eds.; Society of Nematologists: Hyattsville, Maryland, 1987, pp 448–454.
3. Thomason, I. J. In *Vistas on Nematology*; Veech, J. A.; Dickson, D. W., Eds.; Society of Nematologists: Hyattsville, Maryland, 1987, pp 469–476.

4. Sasser, J. N.; Freckman, D. W. In *Vistas on Nematology*; Veech, J. A.; Dickson, D. W., Eds.; Society of Nematologists: Hyattsville, Maryland, 1987, pp 7–14.
5. Comley, J. C. W. *Trop. Med. Parasit.* 1990, *41*,1.
6. Feldmesser, J.; Kochansky, J.; Jaffe, H.; Chitwood, D. In *Agricultural Chemicals of the Future*; Hilton, J. L., Ed; Rowman and Allanheld: Totowa, New Jersey, 1985; pp 327–344.
7. Taylor, C. E.; Murant, A. F. *Nematologica* 1967, *12*, 488.
8. Chang, L.; Rohde, R. A. *Phytopathology* 1969, *59*, 398.
9. Miller, P. M.; Turner, N. C.; Tomlinson, H. *J. Nematol.* 1973, *5*, 173.
10. Sukul, N. C.; Das, P. K.; De, G. C. *Nematologica* 1974, *20*, 187.
11. Husain, S. I.; Masood, A. *Geobios* 1975, *2*, 74.
12. Husain, S. I.; Masood, A. *Acta Bot. Ind.* 1975, *3*, 142.
13. Pillai, S. N.; Desai, M. *Pesticides* 1975, *9*(*4*), 37.
14. Lal, A.; Yadav, B. S.; Nandwana, R. P. *Ind. J. Mycol. Plant Pathol.* 1977, *7*, 68.
15. Ueno, Y.; Iyatomi, K. 1978. *Sci. Rep. Fac. Agr. Meijo Univ.* 1978, *14*, 7.
16. Ram Nath, M. N.; Khan, R. S.; Kamalwanshi, R. S.; Dwivedi, R. *Ind. J. Nematol.* 1982, *12*, 205.
17. Nandal, S. N.; Bhatti, D. S. *Ind. J. Nematol.* 1983, *13*, 123.
18. Bala, S. K.; Sukul, N. C. *Nematrópica* 1987, *17*, 219.
19. Khan, F. A. *Rev. Nématol.* 1990, *13*, 463.
20. Chitwood, D. J. In *Vistas on Nematology*; Veech, J. A.; Dickson, D. W., Eds.; Society of Nematologists: Hyattsville, Maryland, 1987, pp 122–130.
21. Barker, G. C.; Chitwood, D. J.; Rees, H. H. *Invert. Reprod. Devel.* 1990, *18*, 1.
22. Chitwood, D. J. *J. Nematol.* 1990, *22*, 598.
23. Uhlenbroek, J. H.; Bijloo, J. D. *Rec. Trav. Chim. Pays-Bas* 1958, *77*, 1004.
24. Uhlenbroek, J. H.; Bijloo, J. D. *Rec. Trav. Chim. Pays-Bas* 1959, *78*, 382.
25. Gommers, F. J. *Helminthol. Abstr.* 1981, *50B*, 9.
26. Gommers, F. J.; Bakker, J. In *Diseases of Nematodes,* Poinar, G. O., Ed.; CRC Press: Boca Raton, Florida, 1988, Vol. I; pp 3–22.
27. Bijloo, J. D. *Nematologica*, 1965, *11*, 643.
28. Fassuliotis, G.; Skucas, G. P. *J. Nematol.* 1969, *1*, 287.
29. Matsuda, K.; Kimura, M.; Komai, K.; Hamada, M. *Agri. Biol. Chem.* 1989, *53*, 2287.
30. Matsuda, K.; Yamada, K.; Kimura, M.; Hamada, M. *J. Agri. Food Chem.* 1991, *39*, 189.
31. Onda, M.; Takiguchi, K.; Hirakura, M.; Fukushima, H.; Akagawa, M.; Naoi, F. *Nippon Nageikaguku Kaishi* 1965, *39*, 168.
32. Onda, M.; Abe, K.; Yonezawa, K.; Esumi, N.; Suzuki, T. *Chem. Pharm. Bull.* 1970, *18*, 435.
33. Allen, E. H.; Feldmesser, J. *Phytopathology* 1970, *60*, 1013.
34. Allen, E. H., Feldmesser, J. *J. Nematol.* 1971, *3*, 58.

35. Gommers, F. J. *Nematicidal principles in Compositae*. Dissertation, Agric. Univ., Wageningen, 1973; pp 1–73.
36. Kogiso, S.; Wada, K.; Munakata, K. *Agri. Biol. Chem.* 1976, *40*, 2085.
37. Kogiso, S.; Wada, K.; Munakata, K. *Tetrahedron Lett.* 1976, *2*, 109.
38. Kawazu, K.; Nishii, Y.; Nakajima, S. *Agri. Biol. Chem.* 1980, *44*, 903.
39. Kimura, Y.; Mori, M.; Suzuki, A.; Kobayashi, A. *Agri. Biol. Chem.* 1981, *45*, 2915.
40. Mori, M.; Hyeon, S.-B.; Kimura, Y.; Suzuki, A. *Agri. Biol. Chem.* 1982, *46*, 309.
41. Munakata, K. In *Natural Products for Innovative Pest Management*, Whitehead, D. L.; Bowers, W. S., Eds.; Pergamon Press: Oxford, 1983; pp 299–310.
42. Tarjan, A. C.; Cheo, P. C. *Nematocidal Value of some Fatty Acids*. Univ. Rhode Is. Agri. Exp. Sta. Bull. 332, Kingston, 1976; pp 1–41.
43. Sayre, R. M.; Patrick, Z. A.; Thorpe, H. A. *Nematologica* 1965, *11*, 263.
44. Kimura, Y.; Mori, M.; Hyeon, S.-B.; Suzuki, A.; Mitsui, Y. *Agri. Biol. Chem.* 1981, *45*, 249.
45. Osman, A. A.; Viglierchio, D. R. *Rev. Nématol.* 1988, *11*, 93.
46. Viglierchio, D. R.; Wu, F. F. *Nematrópica* 1989, *19*, 75.
47. Malik, M. S.; Sangwan, N. K.; Dhindsa, K. S.; Verma, K. K.; Bhatti, D. S. *Pesticides* 1987, *21*(5), 30.
48. Sangwan, N. K.; Verma, B. S.; Verma, K. K.; Dhindsa, K. S. *Pestic. Sci.* 1990, *28*, 331.
49. Rohde, R. A. *Annu. Rev. Phytopathol.* 1972, *10*, 233.
50. Kaplan, D. T.; Davis, E. L. In *Vistas on Nematology*; Veech, J. A.; Dickson, D. W., Eds.; Society of Nematologists: Hyattsville, Maryland, 1987, pp 267–276.
51. Scheffer, F.; Kickuth, R.; Visser J. H. *Z. Pflanzenahrung Bodenk.* 1962, *98*, 114.
52. Kaplan, D. T.; Keen, N. T. *Rev. Nématol.* 1980, *3*, 123.
53. Veech, J. A. In *Plant Parasitic Nematodes*; Zuckerman, B. M.; Rohde, R. A., Eds.; Academic Press: New York, 1981; Vol. III, pp 377–403.
54. Veech, J. A. 1982. *J. Nematol.* 1982, *14*, 2.
55. Giebel, J. 1982. *Annu. Rev. Phytopathol.* 1982, *20*, 257.
56. Huang, J.-S. In *An Advanced Treatise on Meloidogyne;* Sasser, J. N.; Carter, C. C., Eds.; North Carolina State Univ. Graphics: Raleigh, 1985; Vol. I, pp 165–174.
57. O'Neill, M. J. *Parasitol. Today* 1986, *2*, 358.
58. Rich, J. R.; Keen, N. T.; Thomason, I. J. *Physiol. Plant Pathol.* 1977, *10*, 105.
59. Veech, J. A.; McClure, M. A. *J. Nematol.* 1977, *9*, 225.
60. Veech, J. A. *J. Nematol.* 1979, *11*, 240.
61. Kaplan, D. T.; Keen, N. T.; Thomason, I. J. *Physiol. Plant Pathol.* 1980, *16*, 309.
62. Kaplan, D. T.; Keen, N. T.; Thomason, I. J. *Physiol. Plant Pathol.* 1980, *16*, 319.

63. Zinovieva, S. V.; Chalova, L. I. *Helminthologia* 1987, *24*, 303.
64. Ellenby, C. *Ann. Appl. Biol.* 1951, *38*, 859.
65. Rohde, R. A.; Jenkins, W. R. *Basis for Resistance of Asparagus officinalis var. altilis L. to the Stubby-root Nematode Trichodorus christiei Allen 1957.* Univ. Md. Agri. Exp. Sta. Bull. A-97, College Park, 1958; pp 1–19.
66. Takasugi, M.; Yachida, Y.; Anetai, M.; Masamune, T.; Kegasawa, K. *Chem. Lett.* 1975, *1975*, 43.
67. Meher, H. C.; Walia, S.; Sethi, C. L. *Ind. J. Nematol.* 1988, *18*, 244.
68. Jacobson, M. In *1988 Focus on Phytochemical Pesticides, Vol. I, The Neem Tree;* Jacobson, M., Ed.; CRC Press: Boca Raton, Florida, 1989, pp 133–153.
69. Smith, S. L.; Mitchell, M. J. *Biochem. Biophys. Res. Commun.* 1988, *154*, 559.
70. Barker, G. C.; Mercer, J. G.; Svoboda, J. A.; Thompson, M. J.; Rees, H. H.; Howells, R. E. *Parasitology*, 1989, *99*, 409.
71. Gommers, F. J. *Phytochemistry* 1971, *10*, 1945.
72. Mahajan, R.; Singh, P.; Bajaj, K. L.; Kalsi, P. S. *Nematologica*, 1986, *32*, 119.
73. Prot, J.-C.; Kornprobst, J.-M. *C. R. Acad. Sci. Sér. III,* 1983, *296*, 555.
74. Kogiso, S.; Wada, K.; Munakata, K. *Agri. Biol. Chem.* 1976, *40*, 2119.
75. Tada, M.; Hiroe, Y.; Kiyohara, S.; Suzuki, S. *Agri. Biol. Chem.* 1988, *52*, 2383.
76. Fukuzawa, A.; Furusaki, A.; Ikura, M.; Masamune, T. J. Chem. Soc. Chem. Commun. 1985, *1985*:222,748.
77. Fukuzawa, A.; Matsue, H.; Ikura, M.; Masamune, T. *Tetrahedron Lett.* 1985, *26*, 5539.
78. Miwa, A., Nii, Y.; Okawara, H.; Sakakibara, M. *Agri. Biol. Chem.* 1987, *51*, 3459.
79. Davis, E. L.; Kaplan, D. T.; Dickson, D. W.; Mitchell, D. J. *J. Nematol.* 1989, *21*, 219.
80. Marban-Mendoza, N.; Jeyaprakash, A.; Jansson, H.-B.; Damon, R. A.; Zuckerman, B. M. *J. Nematol.* 1987, *19*, 331.

RECEIVED August 12, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch022

Chapter 22

Utilizing Derivatives of Microbial Metabolites and Plant Defenses To Control Diseases

H. V. Morton[1] and R. Nyfeler[2]

[1]Ciba-Geigy Corporation, P.O. Box 18300, Greensboro, NC 27419
[2]Ciba-Geigy, Ltd., Basle, Switzerland

The phenylpyrroles are a new class of agricultural fungicides related to the natural antibiotic pyrrolnitrin, which is produced by Pseudomonas spp. Pyrrolnitrin fulfills the main requirements of natural products, namely it has interesting biological activity and possesses a simple enough structure to allow the synthesis of analogues. A second area offering a new approach to disease control is that of systemic acquired resistance (SAR) utilizing chemicals with no direct bactericidal or fungicidal effect. Agents which induce SAR include the rice blasticide probenazole and the immunization compound 2,6-dichloroisonicotinic acid and its ester.

Opportunities for Natural Products

A question frequently heard today is: "What is the role of chemicals in the future of crop protection?" We view our business objectives as inseparable from our environmental objectives and thus integrated pest management (IPM) is one of our goals. These are fundamental elements that help drive the discovery, development, production, and sale of our products. The following discussion illustrates the challenges and opportunities for (a) using natural products or their derivatives, and how these may be optimized, and (b) the use of chemicals to stimulate the host plant resistance mechanisms. Such strategies offer new approaches to disease control and a possible means to address the growing public concern for the environment. It should also be recognized that IPM will be the primary strategy of pest control and that chemical crop protection remains an integral feature of IPM. To maintain the value of chemicals in crop protection, the following four objectives must be fulfilled.

1. Continue and even accelerate the search for novel compounds which are effective at very low doses; are selective for the target organisms; show a moderate persistence in the environment; and are nontoxic to humans, nontarget organisms, and the environment.
2. Strengthen the search for methods to delay or eliminate the induction and appearance of resistance to crop protection chemicals.
3. Improve application technology, concentrating on optimization of formulations, placement of the products, and timing of applications.
4. Extend and improve the Integrated Pest Management approach.

This paper will address only the first point, namely the search for new chemicals. Such a search is influenced by:

0097–6156/93/0524–0316$06.00/0

1. The availability of relevant biological test systems.
2. A battery of tests to investigate the fate of the compounds in the environment, and to study the metabolism in plants and animals and the toxicological properties.
3. An ample supply of novel compounds.

The quality and relevance of these tests and the diversity of the chemical structures are the key elements to finding safe, effective crop protection compounds.

While a review of crop protection chemistry shows that synthetic chemicals have clearly dominated the natural products, the main reasons to search for natural products as crop protection chemicals are:

1. Natural products provide an alternative source of new structures,
2. Since natural products are formed and degraded in nature, there is a reasonable chance they will fit IPM, and
3. An understanding of the role of secondary metabolites in natural processes can lead to new crop protection principles.

There are two approaches to searching for new secondary metabolites from plants and microorganisms: (a) random screening, and (b) utilizing a biorational approach, e.g., allelochemicals and induced or preformed metabolites which contribute to the resistance of plants against diseases and insects. Typical examples of these would be phytoalexins or insect antifeedants.

Many microorganisms that are evaluated as biocontrol agents owe their activity, at least in part, to secondary metabolites. Isolation and structure determination of these active principles can result in new interesting lead structures. Table I shows an overview of the natural products which have gained some importance in crop protection. Secondary metabolites from microorganisms show a broader spectrum of activity than those derived from plants. A review of the 8th edition of "The Pesticide Manual" shows that there is a total of 14 natural products out of approximately 570 chemicals listed.

Table I. The Direct Use of Natural Products in Crop Protection

Active Against		
Plants	Microorganisms	Insects
	Derived From Plants	
		Nicotine Rotenone Pyrethrins
	Derived From Microorganisms	
Gibberellic Acid Bialaphos	Blasticidin S Cycloheximide Kasugamycin Pimaricin Polyoxins B and D Validamycin Streptomycin	Delta-endotoxin from _Bacillus subtilis_ Avermectin B1

Japan has been a pioneer in finding uses for natural products in crop protection. The most widely used of these are shown in Table II.

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch022

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch022

Table II. Agricultural Antibiotics Used in Crop Protection in Japan

Name	Introduction	Produced By	Tons Sold In 1986
Blasticidins	1962	Streptomyces griseochromogenes	11
Kasugamycin	1965	S. kasugaensis	91
Polyoxins	1968	S. cacaoi	76
Validamycin	1972	S. hygroscopicus	172
Streptomycin	1957	S. griseus	175

Reference: Noyaku Yoran *(1)*

With the exception of streptomycin, all of these compounds have been discovered and developed in Japan.

It is worth noting that all of these antibiotics are produced by fermentation from Streptomyces species. One of the main reasons these products are not used outside of Japan is that the purity of the products, respective to the by-products, is not sufficient to satisfy the stringent western registration guidelines. EPA requires that any by-product that is present at more than 0.1% of the total amount has to be identified.

The following is an example of a project CIBA-GEIGY undertook using a natural product as a lead structure. Pyrrolnitrin, a secondary metabolite from Pseudomonas pyrrocinia, was isolated by Arima, et al., in 1965 *(2)*. This compound showed interesting antifungal activity and was developed as a topical antimycotic for human use. In the late 1970's pyrrolnitrin was found to be highly active against a range of phytopathogenic fungi in our fungicide screen. The interesting biological activity, combined with an apparently simple structure, made pyrrolnitrin an ideal lead structure for optimization (Figure 1).

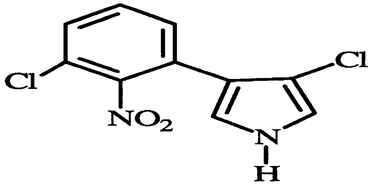
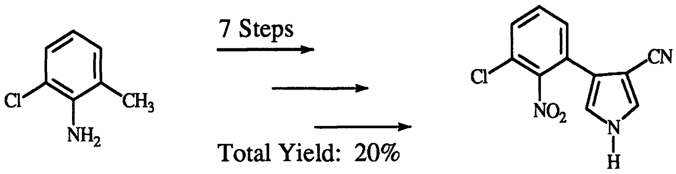


Figure 1. Pyrrolnitrin

Despite the simple looking structure, the compound proved to be quite a challenge for the chemists. The best total synthesis of pyrrolnitrin still required seven steps and afforded the product in only 20% total yield from the reaction sequence (Figure 2). Fortunately, we have discovered a novel procedure to prepare new analogues of pyrrolnitrin. From readily available starting materials, the desired products were prepared in a single step and with good yields. Out of a large optimization program in which the substitutes X and E (Figure 2) were broadly varied, there resulted a number of highly active fungicides belonging to the phenylpyrrole chemical class.

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch022

(a) The Production of Pyrrolnitrin: *(3)*



(b) Analogues with New Synthetic Method: *(4)*

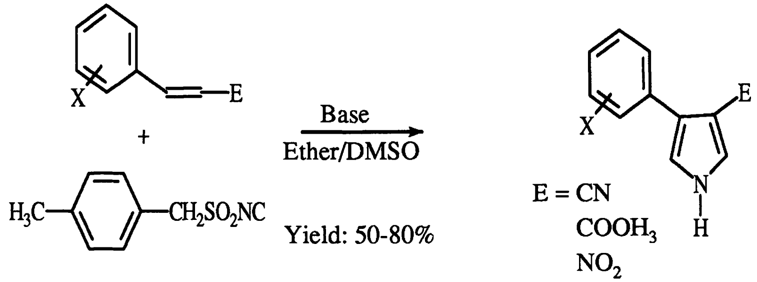


Figure 2. Synthetic Approaches to Pyrrolnitrin Production

The phenylpyrrole fungicides are protectant fungicides with a broad spectrum of activity which allows them to be used as both seed treatments and foliar fungicides. Fenpiclonil (see Table IV) has recently been introduced as a cereal seed treatment in Europe. In the U.S., we are developing a more active analogue, CGA-173506 4-(2,2-difluoro-1, 3-benzodioxol-4-yl) pyrrole-3-carbonitrile (Figure 3, Table III).

Chemical class: Phenylpyrrole

Chemical name: 4-(2,2-difluoro-1,3-benzodioxol-4-yl) pyrrole-3-carbonitrile

Structural formula:

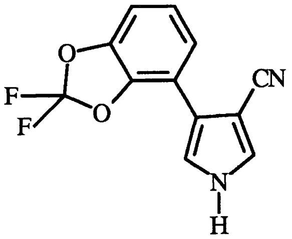


Figure 3. CGA-173506: A new phenylpyrrole fungicide with broad-spectrum disease control

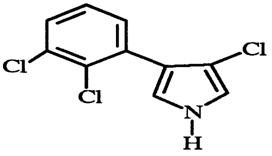
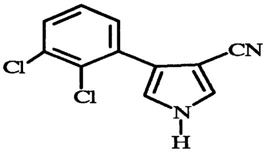
Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch022

Table III. In Vitro Spectrum of Activity of CGA-173506

Fungal Pathogen	EC 50 (ppm a.i.)
Oomycetes:	
Phytophthora infestans	>100
Pythium ultimum	>100
Aphanomyces laevis	10.3
Ascomycetes:	
Ophiobolus graminis	0.18
Monilinia fructicola	0.07
Venturia inaequalis	6.05
Basidiomycetes:	
Rhizoctonia solani	0.04
Pellicularia sasakii	0.10
Sclerotium rolfsii	0.22
Deuteromycetes:	
Helminthosporium teres	0.05
Alternarnia solani	0.15
Botrytis cinerea	0.02
Fusarium culmorum	0.18
Cercospora arachidicola	0.20

With its spectrum and degree of activity, protectant mode of action, and limited toxicity, CGA-173506 is a major breakthrough. The key to this optimization program was the marked increase in light stability of the cyanopyrroles (See Table IV).

Table IV. Modification of Pyrrolnitrin for improved Light Stability

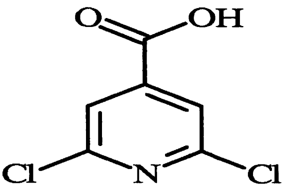
Test	Pyrrolnitrin 	Fenpiclonil 
Light stability		
T 1/2 (suntest- lamp)	27 min	48 hrs.
Botrytis activity		
Greenhouse EC 80	Apple: 6 ppm Bean: 200 ppm	6 ppm 6 ppm
Field activity (100 g/hl)	4%	91%

E. Stamm, CIBA-GEIGY, unpublished

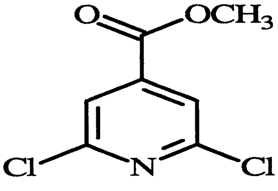
New Crop Protection Principles

Now we will turn to a somewhat more speculative approach: regulation of host parasite interactions, in particular the phenomenon of systemic acquired resistance (SAR) first described by Ross *(5)* on tobacco. In a variety of plant species, the development of necrotic lesions in response to pathogen infection leads to induction of generalized disease resistance in infected tissues. SAR is characterized by the development of a disease-resistant state in plants that have reacted hypersensitively to previous infection by tobacco mosaic virus. Salicylic acid has been implicated as the endogenous signal that activates the resistant state Malamy J. et al. *(6)*

At CIBA-GEIGY we have been trying to synthesize compounds which will induce local and systemic resistance in plants. 2,6-dichloroisonicotinic acid (CGA-41396) and its ester (CGA-41397) are examples of chemistries which induce host plant resistance (Figure 4). Ward, et al. *(7)* have shown that these products also induce SAR gene expression by following nine classes of RNAs (by gel blot hybridization) that are coordinately induced concomitantly with the onset of SAR.



CGA-41396



CGA-41397

Figure 4. Chemical structures of isonicotinic acid derivatives

The onset of SAR is characterized by a decrease in the size of lesions of foliar pathogens. This induction response takes about six days to develop in the case of TMV in tobacco as described by Ross *(4)*. Isonicotinic acid was shown by Ward, et al. *(7)* to impact lesion size within four days and reached a maximum of steady-state RNA at six days after infection.

Practical experience in field trials has shown these isonicotinic acids must be applied preventively to provide acceptable disease control. It is of interest to note that monocotyledonous plants express this resistance for longer than dicotyledonous plants, which require applications every seven to ten days to maintain the expression of induced resistance. These products are effective against bacteria, fungi, and viruses, as well as Pyricularia oryzae (rice blast) via seed-treatment. The isonicotinic acid derivatives perform best against the powdery and downy mildews, while they are weaker against thymatrophic parasites (those that kill tissues in advance of their own cells and then invade the killed tissues) such as Rhizoctonia solani and Septoria spp.

Unfortunately the amount of phytotoxicity associated with foliar sprays of CGA-41396 and CGA-41397 precludes their commercial development. CIBA-GEIGY is currently working with a second generation of products which are safer to plants. There are several opportunities offered by products which result in SAR. One of these possibilities is for plant breeders to use such products to accentuate host plant resistance to plant pathogens. We view this chemistry as resulting in exciting new forms of disease control in the future.

Literature Cited

1. Noyaku Yoran, **1986** and **1988** published by Japan Plant Protection Association.
2. Arima, K; Imanaka, H; Kousaka, M; Fukuda, A; Tamura, G. J. Antibiotics **1965**, 18, 211-219.

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch022

3. Gosteli, J. Helv. Chim. Acta **1972**, 55, 451-460.
4. Van Leusen, A. M; Siderius, H; Hoogenboom, B. E; van Leusen, D. Tettrahed. Letters **1972**, 5337-5340.
5. Ross, A. F. Virology **1961**, 14, 340-358.
6. Malamy, J; Carr, J. P; Klessig, D. F; and Raskin, I. Science **1990**, 1002-1004.
7. Ward, E. R; Uknes, S. J; Williams, S. C; Dincher, S. S; Winderhold, D. L; Alexander, D. C; Ahl-Goy, P; Metraux, J. P; and Ryals, J. A. Plant Cell **1991**, 3, 1005-1094.

RECEIVED September 1, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch022

Chapter 23

Strategies and Techniques for Improving Biocontrol of Soilborne Plant Pathogens

D. P. Roberts and D. R. Fravel

Biocontrol of Plant Diseases Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705

For successful biocontrol, timing and placement of inoculum of biocontrol agents can be more important than the population size of the biocontrol agent. Delivery of the biocontrol agent is often timed so that it coincides with a period of low biological diversity and maximum pathogen accessibility. Control strategies include: 1) protection of the root or seed; 2) impeding the progress of the pathogen; and 3) destruction of pathogen inoculum. In addition to being in the right place at the right time, the biocontrol agent must also express specific traits at the appropriate time. Traits associated with biocontrol agents include competition for nutrients and production of antibiotics and enzymes.

Environmental and food safety issues have fostered increased interest in alternatives to chemical pesticides for control of soilborne plant pathogens. Disease control strategies involving the use of introduced, beneficial bacteria and fungi (biocontrol agents) for control of soilborne, plant pathogenic bacteria and fungi can be integrated into pest management strategies using minimal quantities of chemical pesticides. In some cases, the biocontrol agents may replace chemical pesticides. In this chapter we discuss current areas of research aimed at enhancing the effectiveness of these biocontrol strategies. Examples are given to illustrate the status of this research and the experimental approaches used in this research. This chapter is not intended as an all-inclusive review of the literature.

Elements of Successful Biocontrol

Biocontrol of soilborne plant pathogens is dependent on establishing a sufficient population of active propagules of the biocontrol agent in the right place at the right time. Expression of the appropriate traits by the biocontrol agent, timing

This chapter not subject to U.S. copyright
Published 1993 American Chemical Society

of application of the biocontrol agent, and placement of the biocontrol agent can be more important to the success of biocontrol than the population size of the biocontrol agent. Also, understanding the ecology of the biocontrol agent, the pathogen, the plant and the interactions among these organisms is important in determining how, when, and where to introduce the biocontrol agent. Hence, areas of intensive research include: 1) development of control strategies based on the ecology and epidemiology of the biocontrol interactions and 2) elucidating the traits expressed by the biocontrol agent that are necessary for biocontrol and optimizing their expression.

Biocontrol agents disrupt the disease process by inactivating, retarding growth and movement, or killing the pathogen. Several strategies have been used to achieve biocontrol of soilborne plant pathogens. This chapter discusses three of these strategies: protection of the rhizosphere or spermosphere, impeding advancement of the pathogen through soil, and destruction of pathogen inoculum.

Biocontrol Strategies

Protection of the Rhizosphere or Spermosphere. Protection of the rhizosphere (zone of root influence) or spermosphere (zone of seed influence) is an attractive strategy for several reasons. First, delivery of the biocontrol agent is greatly simplified. The biocontrol agent can be applied to the seed as a seed treatment prior to planting for protection of the spermosphere (5, 27) or to the root as a root dip for protection of the rhizosphere (14). Both treatments are amenable for use in many crop production strategies. Second, biocontrol can be achieved with small quantities of biocontrol agent inoculum delivered to the seed or root surface. Since the pathogen must contact the plant for infection to occur, the biocontrol agent has been properly positioned to control the disease. There is no need to apply large quantities of the biocontrol agent to the bulk soil. In addition, the plant deposits a variety of reduced carbon compounds and other nutrients in the soil through root or seed exudation and through sloughing off of root cells and the seed coat. These nutrients stimulate growth and proliferation of the biocontrol agent in the rhizosphere or spermosphere.

One example of successful biocontrol by protection of the rhizosphere is the use of Agrobacterium radiobacter strain 84 (strain 84) to control crown gall caused by pathogenic Agrobacterium radiobacter var. tumifaciens. The pathogen produces a tumor-like gall at the soil line of many hosts, particularly rosaceous hosts such as almond, peach and rose. Strain 84 is usually supplied in a dry carrier such as peat. For use, the formulated material is wetted with chlorine-free water and bare-rooted plants are dipped in the suspension just prior to being transplanted in the field (14).

Retarding Pathogen Growth Through Soil. Retarding growth of the pathogen through soil has also been used as a strategy for biocontrol of soilborne plant pathogens. Generally, roots grow through soil and contact pathogen propagules since pathogen growth through raw soil is limited by microbial competition for nutrients. In soil that has been disinfested, by heating or with chemicals, introduced pathogens spread through the soil quickly due to the absence of competition for nutrients.

The low biological diversity in soil created by disinfestation presents an opportunity for biocontrol, such as in the control of Fusarium wilt of chrysanthemums in the greenhouse. Greenhouse-grown chrysanthemums are often grown in freshly steamed potting mix. Fans for greenhouse cooling draw in large volumes of air which can contain air-borne spores of the pathogen Fusarium oxysporum f. sp. chrysanthemi. This leads to the introduction and establishment of Fusarium in the disinfested potting mix. Success of biocontrol in this strategy depends on the biocontrol agent proliferating in the freshly steamed potting mix and utilizing the nutrients that would otherwise be available to the pathogen. The addition of Trichoderma viride strain T-1-R9 to freshly steamed potting mix resulted in increased CO_2 evolution during the first 12 days which indicated an increased level of nutrient utilization. There was also a reduction in the final population density of F. oxysporum f. sp. chrysanthemi and in disease incidence (24).

Destruction of Pathogen Inoculum. Another strategy that has been used successfully for biocontrol of plant diseases is the destruction of overwintering inoculum of the pathogen. Destruction of overwintering inoculum is feasible because: 1) biocontrol agents are living organisms and, hence, are capable of reproduction, growth, and spread; 2) in soil, pathogens occur in aggregate distribution in association with the crop plants or plant residues (facilitating spread of the biocontrol agent); and 3) inoculum of some soilborne pathogens is formed above ground and is therefore accessible to applications of the biocontrol agent.

Sporidesmium sclerotivorum, a mycoparasite of the plant pathogen Sclerotinia minor, can provide complete control of Sclerotinia lettuce drop in the field at application rates as low as 0.2 kg of the formulated biocontrol agent/ha (1). Pathogen sclerotia (overwintering structures) are formed in the above ground portion of the lettuce crop. When this crop residue is disked into the soil, sclerotia remain in an aggregate distribution in the soil. Therefore, if one sclerotium in the aggregate is infected with S. sclerotivorum, the biocontrol agent can spread to destroy all sclerotia in the aggregate, resulting in disease control.

Introduction of the Biocontrol Agent

In addition to considering which strategy would be most appropriate for a particular crop-pathogen situation, it is also necessary to consider where and when introduction of the biocontrol agent is most likely to result in successful biocontrol. Soil is a complex physical, chemical, and biological environment that is constantly evolving (7). Nutrient sources in soil are limiting to growth and reproduction of soil microbes. Thus, chances for successful biocontrol can be increased by introducing the biocontrol agent at a time and/or place where the environment is biologically less complex or microbial competition is lower. This can be a time and place that occurs naturally during the growing cycle, or it can be a special environment that is created for the purpose of introducing the biocontrol agent. Different levels of ecological complexity are associated with various agricultural systems. Less ecologically complex agricultural systems, such as greenhouse crops grown in potting mix or steamed soil, are often more amenable to biocontrol than field situations (23). In some systems, such as the biocontrol of Fusarium wilt of chrysanthemum discussed previously, biocontrol agents are introduced to freshly heat-sterilized or fumigated soil. Heat sterilization and fumigation of the soil kills resident organisms leading to a situation of reduced ecological complexity.

Establishment of biocontrol agents may be enhanced through formulation with nutrient sources that are uniquely available to the biocontrol agent. For example, a number of inexpensive carbon sources such as L-arabitol, D-arabinose, L-xylose, xylitol, and L-mannose are not commonly found in nature and are not utilized by most microbes (25). Biocontrol agents that can utilize these uncommon compounds, or spontaneous mutants of biocontrol agents that have acquired the ability to grow on these uncommon carbon sources, could be added to the soil along with these carbohydrates. Microorganisms probably have not evolved the catabolic pathways specific for compounds they have not encountered in their environment (25). Thus, competition for these uncommon compounds should be less than competition for commonly encountered carbon sources.

Traits Associated with Biocontrol

The biocontrol agent must express certain traits for biocontrol to occur. It is necessary to identify these traits and to determine how the environment modifies the expression of these traits before the performance of biocontrol agents can be predicted or enhanced (Nelson, E.B; Maloney, A.P. Can. J. Plant Pathol., in press). Techniques from analytical chemistry, biochemistry, and molecular biology are being used to identify the traits expressed by biocontrol agents that result in biocontrol. These techniques are used 1) to demonstrate that the trait is expressed in situ and 2) to demonstrate that a correlation exists between the expression of the trait and biocontrol. The examples given below illustrate how these techniques have been used in this evaluation process.

Antibiotics Involved in Biocontrol. Phenazine-producing pseudomonads suppress primary infections and limit secondary spread of Gaeumannomyces graminis var. tritici, the fungus causing take-all of wheat (4). Thomashow et al. (39) demonstrated that the antibiotic phenazine-1-carboxylate was produced by derivatives of Pseudomonas fluorescens strain 2-79 in wheat rhizospheres. This antibiotic was detected in wheat rhizospheres colonized by strain 2-79RN10, a rifampicin-resistant derivative of strain 2-79. The antibiotic was not detected in wheat rhizosphere colonized by strain 2-79-B46, a phenazine-1-carboxylate deficient mutant of strain 2-79. In addition, this antibiotic was detected in wheat rhizospheres colonized by a derivative of strain 2-79-B46 which had phenazine-1-carboxylate production restored by genetic complementation (39). Strong evidence for a role for this antibiotic in biocontrol by P. fluorescens strain 2-79 comes from biocontrol assays with random Tn5 mutants of this strain. Six mutants, each containing a single transposon Tn5 insertion, were deficient in phenazine-1-carboxylate production and had reduced biocontrol capabilities when compared to the parental strain (38). Thomashow and coworkers (39) also demonstrated a role for phenazine-1-carboxylate in biocontrol in this system by detecting production of this antibiotic in situ and correlating production of this antibiotic with biocontrol.

The beneficial fungus Gliocladium virens controls damping-off of zinnia, cotton, and cabbage caused by the pathogenic fungus Pythium ultimum in nonsterile soilless mix (21). An antibiotic produced by G. virens may have a role in this biocontrol interaction. Size-fractionated culture supernatants from G. virens containing compounds that were < 10 kDa in size inhibited P. ultimum sporangial germination and mycelial growth in vitro. The antibiotic gliotoxin was the only compound in these < 10 kDa preparations that was inhibitory to P. ultimum (31). Gliotoxin has been detected in a variety of soils and soilless media colonized by G. virens (22). In addition, quantities of gliotoxin in the soil were related to the level of biocontrol of damping-off. Damping-off was significantly suppressed in peat moss-vermiculite medium, composted mineral soil, and clay soil but not in a sandy soil. Quantities of gliotoxin detected in these soils were 0.42, 0.36, 0.20, and 0.02 µg/cm3, respectively (22).

Enzymes Involved in Biocontrol. Glucose oxidase, excreted by the fungal biocontrol agent Talaromyces flavus, catalyzes the production of hydrogen peroxide from glucose (15). Microsclerotia (overwintering structure) of the plant pathogen Verticillium dahliae are killed by hydrogen peroxide in vitro (15). Glucose oxidase purified from culture filtrates of T. flavus Tf1, when applied to sand columns reduced the growth rate of V. dahliae when eggplant roots were present in the sand columns (9). In addition, no effect on growth rate of V. dahliae was evident in the absence of eggplant roots in this experiment. This suggests that glucose, the substrate used in the production of hydrogen peroxide, is supplied by exudates from the eggplant root. In addition, the experiment demonstrates that the enzyme glucose oxidase can contribute to biocontrol. Another line of evidence for the involvement of glucose oxidase in biocontrol comes from a study using a natural variant of the parental isolate T. flavus Tf1. The single-spore isolate Tf1-np produced only 2% of the level of glucose oxidase produced by Tf1, while Tf1-np was similar to Tf1 for all other characteristics measured. Isolate Tf1 provided control of Verticillium wilt of eggplant while Tf1-np did not control the disease (9).

Chitinolytic strains of S. marcescens reduce the incidence of disease caused by Sclerotium rolfsii (30) and Fusarium oxysporum f.sp. pisi (11). Chitinase, secreted by the Gram-negative soil bacterium Serratia marcescens (32), catalyzes the degradation of the fungal cell wall component chitin. A partially purified preparation of chitinase, isolated from cultures of an E. coli strain containing a cloned S. marcescens chitinase gene, caused rapid bursting of S. rolfsii hyphal tips (35). In addition, application of this chitinase preparation to bean plants reduced the incidence of disease caused by S. rolfsii (35). Jones et al. (11) demonstrated that a chitinase gene was involved in biocontrol of F. oxysporum f.sp. pisi by S. marcescens strain QMB1466. The chiA gene, which encodes one of the chitinases produced by this strain, was cloned and subsequently mutated by insertional inactivation with an antibiotic resistance gene. A strain that produced reduced levels of chitinase was constructed by incorporating this mutated chiA gene into the S. marcescens chromosome. The resultant strain, which produced reduced levels of chitinase, did not control Fusarium yellows of pea as well as the parental strain (11) correlating chitinase production by S. marcescens with biocontrol. In another set of experiments a chitinase gene from S. marcescens was mobilized into Pseudomonas fluorescens strain NRRL B-15135 by conjugation (37). The resultant strain, P. fluorescens NRRL B-15135 (pLES1), had enhanced biocontrol properties (37). Collectively these experiments indicate that chitinase is involved in biocontrol by S. marcescens and that addition of this trait to non-chitinolytic biocontrol agents can enhance biocontrol performance.

Nutrient Competition Involved in Biocontrol. Competition for various nutrients is also involved in biocontrol. Competition for iron between plant growth-promoting rhizobacteria (PGPR) (16) and plant pathogens has been particularly well documented (19). A number of experimental approaches implicate siderophore-induced iron limitation as a mechanism involved in biocontrol. Strains of PGPR, including Pseudomonas fluorescens, P. putida and some closely related strains, produce siderophores in culture which have a high affinity for soluble ferric iron (Fe^{+3}). Pyoverdine, a type of siderophore, inhibits growth of a number of plant pathogens on agar plates (17, 18). Available iron in soil has been correlated with biocontrol. When iron availability was enhanced with various soil amendments, plant disease suppression was not present. However, in nonamended soils there was plant disease suppression (17, 18, 33). Soil amended with a purified pyoverdine, pseudobactin, or a synthetic iron chelating agent, EDDHA, mimicked plant disease suppression by a fluorescent pseudomonad. Finally, experiments with well characterized near-isogenic strains correlated pyoverdine production with biocontrol in several biocontrol interactions. Pyoverdine deficient strains containing single transposon insertions were reduced in their biocontrol capabilities. This was the case for P. putida strain WCS controlling minor pathogens of potato (3, 34) and P. fluorescens 3551 controlling Pythium damping-off of cotton (20).

The experiments outlined above indicate that the production of antibiotics, enzymes, and siderophores are traits associated with some biocontrol interactions. Some other traits thought to be involved in biocontrol are listed in Table I.

Multiple Traits Involved in Biocontrol. Current research findings indicate that biocontrol is not due to the expression of a single trait by the biocontrol agent. It is thought that biocontrol is a complex polygenic phenotype resulting from the expression of a variety of traits concurrently or in sequence (Nelson, E.B.; Maloney, A.P. Can. J. Plant Pathol., in press). Evidence supporting this hypothesis comes from genetic analyses of a number of different interactions involving biocontrol bacteria.

Table I. Examples of Traits Associated with Biocontrol

Biocontrol Agent	*Trait*	*Reference*
Agrobacterium radiobacter	production of Agrocin 84	6, 13, 29
Enterobacter cloacae	production of ammonia	10
Enterobacter cloacae	binding to pathogen hyphae	26
Enterobacter cloacae	blocking signal for pathogen germination	28
Gliocladium virens	production of gliotoxin	22, 31
Pseudomonas fluorescens	production of phenazine-1-carboxylate	38, 39
Pseudomonas fluorescens	production of 2,4-diacetylphloroglucinol	12
Pseudomonas fluorescens	production of hydrogen cyanide	40
Pseudomonas fluorescens	production of siderophore	20
Serratia marcesens	production of chitinase	11
Talaromyces flavus	production of glucose oxidase	9

With the agrobacterium system, the transfer of genes encoding agrocin 84 (a disubstituted fraudulent adenine nucleotide analogue) into isolates of A. radiobacter confers the capacity to produce agrocin 84 on the recipient strain. However, very few of the resultant strain constructions control crown gall (8, 36). This suggests that the genetic background within which agrocin 84 is expressed is important. In addition, a derivative of strain A. radiobacter K84 that no longer produces agrocin 84 can control agrocin-84-sensitive and agrocin-84-resistant A. tumefaciens strains under certain experimental conditions.

Similar results are observed with near isogenic strains of P. fluorescens that no longer produce phenazine-1-carboxylate or pyoverdine. In both cases the mutant strains were decreased in biocontrol capability relative to the parental strain. However, these strains still produced significant disease suppression relative to the pathogen control treatment, where no biocontrol agent was applied (20, 38).

Finally, P. fluorescens strain CHA0, which controls black root rot of tobacco, produces a number of metabolites in culture, including hydrogen cyanide, siderophores, and 2,4 diacetylphloroglucinol (Phl) (2, 12). Near-isogenic strains of strain CHA0 deficient in the production of hydrogen cyanide or Phl were constructed by the insertional inactivation of the genes involved in production of these compounds (12, 40). Both deficiencies resulted in a decreased ability to suppress black root rot under gnotobiotic conditions.

In all of the experiments outlined above, the deletion of a trait involved in biocontrol resulted in a decreased biocontrol capability but not a loss of the biocontrol phenotype. This indicates that, at least in the biocontrol systems discussed, biocontrol is the manifestation of the expression of several traits by the biocontrol agent. Unfortunately, the identity of all of the traits expressed by any particular biocontrol agent are not known for any given biocontrol interaction.

Summary

Traits associated with biocontrol of soilborne plant pathogens are understood in only a few systems. Continued study of biocontrol interactions will undoubtedly result in the detection of other unknown, and perhaps unexpected, traits associated with biocontrol. Both the level of biocontrol and the reliability of this control can be improved by understanding how components of the disease setting work and interact at the population, organismal, cellular, and biochemical levels, and how these biological components interact with the environment. This knowledge can be used to make adjustments, exploiting attributes of each component, resulting in improved disease control. Thus, biocontrol can be integrated into current production systems.

Literature Cited

1. Adams, P.B.; Fravel, D.R. Phytopathology 1990, 80, 1120-1124.
2. Ahl, P.; Voisard, C.; Défago, G. J. Phytopathol. 1986, 116, 121-134.
3. Bakker, P.A.H.M.; Lamers, J.G.; Bakker, A.W.; Marugg, J.D.; Weisbeek, P.J.; Schippers, B. Neth. J. Plant Pathol. 1986, 92, 249-256.
4. Bull, C.T.; Weller, D.M.; Thomashow, L.S. Phytopathology 1991, 81, 954-959.
5. Callan, N.W.; Mathre, D.E.; Miller, J.B. Plant Disease 1990, 74, 368-372.
6. Cooksey, D.A.; Moore, L.W. Phytopathology 1982, 72, 919-921.
7. Curl, E.A.; Truelove, B. The Rhizosphere; Springer-Verlag: New York; 1986.
8. Ellis, J. G.; Kerr, A.; van Montagu, M.; Schell, J. Physiol. Plant Pathol. 1979, 15, 311-319.
9. Fravel, D.R.; Roberts, D.P. Biocontrol Sci. Technol. 1991, 1, 91-99.
10. Howell, C.R.; Beier, R.C.; Stipanovic, R.D. Phytopathology 1988, 78, 1075-1078.
11. Jones, J.D.G.; Grady, K.L.; Suslow, T.V.; Bedbrook. J.R. EMBO J. 1986, 5, 467-473.
12. Keel, C.; Schnider, U.; Maurhofer, M.; Voisard, C.; Laville, J.; Burger, U.; Wirthner, P.; Haas, D.; Défago, G. Molec. Plant-Microbe Interactions 1992, 5, 4-13.
13. Kerr, A.; Htay, K. Physiol. Molec. Plant Pathol. 1974, 4, 37-44.
14. Kerr, A. Plant Dis. 1980, 64, 25-30.
15. Kim, K.K.; Fravel, D.R.; Papavizas, G.C. Can. J. Microbiol. 1990, 36, 760-764.
16. Kloepper, J.W.; Schroth, M.N. In Proc. Fourth Int. Conf. Plant Pathol. Bact. Vol. 2, 1978; Station de Pathologie Végétale et Phytobactériologie.
17. Kloepper, J.W.; Leong, J.; Teintze, M.; Schroth, M.N. Nature (London) 1980, 286, 885-886.
18. Kloepper, J.W.; Leong, J.; Teintze, M.; Schroth, M.N. Curr. Microbiol. 1980, 4, 317-320.
19. Leong, J. Annu. Rev. Phytopathol. 1986, 24, 187-209.

20. Loper, J.E. Phytopathology 1988, 78, 166-172.
21. Lumsden, R.D.; Locke, J.C. Phytopathology 1989, 79, 361-366.
22. Lumsden, R.D.; Locke, J.C.; Adkins, S.T.; Ridout, C.J. Phytopathology 1992, 89, 230-235.
23. Marois, J.J.; English, J.T. Integration of biological control of plant pathogens into IPM programs. In Proc National Integrated Pest Management Symposium/Workshop, 25-28 Apr 1989. National IPM Coordinating Committee, 1989, pages 33-40.
24. Marois, J.J.; Locke, J.C. Phytopathology 1985, 75, 115-118.
25. Mortlock, R.P. In Advances in Microbial Physiology Vol 13.; Rose, A.H.; Tempest, D.W., Eds.; Academic Press: New York, 1976, pages 1-53.
26. Nelson, E.B.; Chao, W-L.; Norton, J.M.; Nash, G.T.; Harman, G.E. Phytopathology 1986, 76, 327-335.
27. Nelson, E.B. Plant Disease 1988, 72, 140-142.
28. Nelson, E.B. Plant Soil 1990, 129, 61-73.
29. New, P.B.; Kerr, A. J. Appl. Bacteriol. 1972, 35, 279-287.
30. Ordentlich, A.; Elad, Y.; Chet, I. Phytopathology 1988, 78, 84-88.
31. Roberts, D.P.; Lumsden, R.D. Phytopathology 1990, 80, 461-465.
32. Roberts, R.L.; Cabib, E. Anal. Biochem. 1982, 127, 402-412.
33. Scher, F.M.; Baker, R. Phytopathology 1982, 72, 1567-1573.
34. Schippers, B.; Lugtenberg, B.; Weisbeek, P.J. In Innovative Approaches to Plant Disease Control; Chet, I. Ed.; John Wiley and Sons: NY, 1987.
35. Shapira, R.; Ordentlich, A.; Chet, I.; Oppenheim, A.B. Phytopathology 1989, 79, 1246-1249.
36. Shim, J-S.; Farrand, S.K.; Kerr, A. Phytopathology 1987, 77, 463-466.
37. Sundheim, L.; Poplawsky, A.R.; Ellingboe, A.H. Physiol. Molec. Plant Pathol. 1988, 33, 483-491.
38. Thomashow, L.S.; Weller, D.M.; J. Bacteriol. 1988, 170, 3499-3508.
39. Thomashow, L.S.; Weller, D.M.; Bonsall, R.F.; Pierson, L.S. III. Appl. Envion. Microbiol. 1990, 56, 908-912.
40. Voisard, C.; Keel, C.; Haas, D.; Défago, G. EMBO J. 1989, 8, 351-358.

RECEIVED July 22, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

Chapter 24

Minimizing Nontarget Effects of Fungicides

N. N. Ragsdale[1], M. J. Henry[2], and H. D. Sisler[3]

**[1]National Agricultural Pesticide Impact Assessment Program,
U.S. Department of Agriculture, Washington, DC 20250
[2]Crop Disease Management Discovery Group, DowElanco, P.O. Box 708,
Greenfield, IN 46140
[3]Botany Department, University of Maryland, College Park, MD 20742**

The history of fungicides demonstrates a movement toward compounds that are less likely to produce adverse environmental effects. The primary concerns about fungicide residues have focused on chronic toxicity, carcinogenicity in particular, resulting from dietary exposure. Because of their low acute non-target toxicity, fungicides have not been as actively sought in environmental monitoring as other classes of pesticides; their residues are rarely found in random sampling of soil, water, and air. Effects on microorganisms other than pathogenic fungi are the most likely non-target impacts of fungicide use. Industry is currently taking a proactive approach to discover and develop new products with minimum environmental impact and an improved toxicological profile.

All economically important plants are subject to attack by one or more fungal species. Because fungal infection can cause a great reduction in both yield and product quality, fungicides are used to control plant diseases. Fungicides are applied:

1. to soil to reduce pathogens before planting;
2. to seeds prior to planting to prevent fungal infection;
3. to established plants to prevent fruit, foliar, and root infections;
4. to established plants to eradicate infections; and
5. post harvest to reduce infections that impact storage, shipment, and shelf life.

Fungicides are used in conjunction with non-pesticidal measures such as resistant plant varieties, pruning and burning diseased parts, managing irrigation and drainage methods, and crop rotation. If a resistant crop variety is available or if a disease can be controlled by non-chemical methods, fungicides generally are not used. However, most successful integrated pest management (IPM) programs include the use of fungicides.

0097–6156/93/0524–0332$06.00/0

Without fungicides there would be a substantial increase in crop failures which would impact availability and cost of agricultural products. In recognition of the value of fungicides in production, storage, and distribution, as well as the need to minimize adverse environmental effects, ecological principles should be considered in designing a strategy for disease control. Because fungicides are used in a variety of ways and applied to a variety of sites with varying climatic conditions, a crop production strategy should be developed for a specific situation in order to reduce detrimental impacts on the environment.

Historical Perspective

A general overview of the history of fungicides, using examples of those commonly associated with agricultural practices, will demonstrate a movement toward compounds that are less likely to cause environmental concerns. This trend emphasized the advances made through research that have contributed to a better understanding of pathogenic organisms and the disease process. This information, in turn, is important in the development of fungicides with minimal non-target effects. The selectivity and site specificity of the newer compounds reduce the chances of non-target effects as well as quantities required to achieve disease control. The systemic properties of the new compounds make them more amenable to IPM since they show therapeutic action and can be applied after infection.

Sulfur, the oldest effective fungicide, is still used extensively. It is mentioned as a purifying agent in Homer's "Iliad" and "Odyssey", which were written in the eighth century B.C. The first reference to sulfur as a fungicide appeared in 1802 (*1*). The primary advantages of sulfur are low cost and exemption from tolerance requirements. Detriments include phytotoxicity to a number of plant species, particularly in hot weather, the large application quantities required, and the limited number of diseases controlled (*2*). There are also disadvantages in the fact that sulfur may cause human injury, such as skin and eye irritation (*3*).

A number of heavy metal ions show fungicidal properties; however, only compounds of copper have played a major role as plant protectants while inorganic compounds containing zinc, cadium, or mercury have seen limited use (*2*). Sulfur and the copper-containing Bordeaux mixture were by far the major chemicals used to control plant pathogens from the late 1800's until the synthetic organic compounds were adopted for practical use in the 1940's.

With the introduction of synthetic organic fungicides, the amount of chemicals required for disease control dropped. Thiram, a dialkyldithiocarbamate, was the first of this group; it was developed in 1934 (*4*). The development of the ethylenebisdithiocarbamates (EBDC's), which include zineb, maneb, and mancozeb, followed. Organotins (e.g. triphenyltin compounds), quinones (e.g. dichlone), and phthalimides (e.g. captan) were developed during the 1940's and 1950's. The fungicidal activity of chlorothalonil was reported in the mid-1960's (*2*).

All of these compounds, along with sulfur and the inorganic copper compounds, are classed as broad spectrum, multisite surface protectants. Because they are not absorbed or translocated by the plant, they are suited only for prophylactic use. They must be present on the plant surface when a pathogen arrives. These compounds may damage plants because the margin of safety between phytotoxicity and fungitoxicity may be narrow (*5*). However, because they have multiple sites of fungitoxic action, these fungicides have had few problems with fungal resistance and are extremely valuable for use in resistance management programs designed to prevent or delay fungal resistance associated with the use of newer compounds that are highly specific in their modes of action.

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

A new era in plant disease control emerged with the introduction of the systemic fungicdes in the sixties. In order to be a successful systemic pesticide, a compound must be able to penetrate a plant's natural barriers, which include the waxy cuticle at the leaf surface, the cell wall, and the cell membrane. The fungicide must withstand the plant's metabolism so the toxic moiety is not broken down before it interacts with the pathogen. The compound must demonstrate selective toxicity so it is toxic to the fungus and not the plant (*7*). Systemic fungicides have the advantage over the surface protectants of being internally therapeutic and can cure a disease after infection has occurred. This property makes them particularly valuable in integrated pest management (IPM) programs because it allows a more flexible application schedule and reduces the amount of fungicide required (*8*).

In contrast to the older inorganic fungicides and organic multisite inhibitors, which interact with a number of biochemical targets involved with numerous cellular functions, the systemic compounds show a greater degree of specificity. This specificity, which reduces the risk of non-target effects, increases the risk of selective resistance development in the fungal population. Various resistance strategies have been proposed employing compounds with various modes of action, including the multisite inhibitors.

The carboxamides, which include carboxin, were introduced for practical use in the early 1970's. They are active principally against smut fungi, rust fungi, and *Rhizoctonia solani*, a soil fungus (*11*). These compounds interfere with energy production by blocking the transfer of electrons from succinate to ubiquinone in the mitochondrial electron transport pathway (*12*).

Introduction of the benzimidazole fungicide, benomyl, demonstrated that systemic compounds offered the potential for control of a wide range of plant diseases (*6*). Benomyl and other benzimidazoles bind to the β–tubulin subunit in sensitive organisms and interfere with microtubular dependent processes such as mitosis and hyphal growth (*9*). The benzimidazoles are generally effective for a broad range of plant diseases with the exception of those caused by Oömycetes and Zygomycetes (*10*).

In the mid-1970's a major impact was seen in disease control with the introduction of the systemic fungicides that inhibit sterol biosynthesis. This group encompasses compounds that are effective against a broad range of fungal diseases of humans and other animals as well as plants (*2*). Imazalil, triadimefon, fenarimol, propiconazole, tridemorph, terbinafine, and triforine are in this group, which currently includes more than 30 compounds. Sterol biosynthesis inhibitors may be divided into three groups based on their primary site of action.
These are:

1. squalene epoxidase inhibitors;
2. sterol 14α- demethylase inhibitors; and
3. sterol Δ^8 -Δ^7 isomerase inhibitors and/or sterol 14-reductase inhibitors.

At the present time, agricultural fungicides fall into the second and third groups with the majority in the second. The inhibitory effects result in a lack of key sterols. This leads to a modification of fungal membranes resulting in detrimental or lethal effects (*2*). Compounds in the second group act through interference with a cytochrome P-450 monooxygenase enzyme involved in sterol 14α-demethylation. In some instances, particularly when application rates are exceeded, growth regulatory effects may be noted in higher plants. These are the result of inhibition of a P-450 enzyme involved in gibberellin biosynthesis (*2*).

Other fungicides introduced into practical use since 1970 include fosetyl-Aluminum, the phenylimides and the dicarboximides. Fosetyl-Al is the only commercially available fungicide that is known to be transported in a downward direction in plants, thus permitting control of root diseases by foliar or trunk/stem

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

applications (*13*). The mode of action of fosetyl-Al is not clearly understood, but it is certain that in the plant fosetyl-Al is quickly degraded to phosphonate (phosphonic acid), which is the principle active component. The action of phosphonate almost certainly lies within the pathogen, but the effects also appear to cause an increase in plant resistance to fungal infection (*14*, *15*). The phenylamides, which are specific for Oomycetes, include metalaxyl, furalaxyl, benalaxyl, ofurace, cyprofuram, and oxadixyl. These compounds act through inhibition of ribosomal RNA synthesis (*16*). The dicarboximides (e.g. vinclozolin, iprodione, and procymidone) are used to control a number of agriculturally important diseases. Their primary mode of action is uncertain with effects such as membrane damage and interference with nuclear function as well as cellular motile function considered secondary (*17*). Although a number of studies have indicate the dicarboximides may be translocated, their practical use has been prophylactic as contact fungicides (*18*).

A new approach to disease control is seen in the action of the fungicide, tricyclazole. At concentrations that do not inhibit fungal growth this compound blocks certain reductase reactions in the fungal polyketide pathway to melanin and interferes with appressorial function (2). Melanization of the appressorial wall appears necessary to maintain osmotic forces as well as the architecture and rigidity required for the pathogen to penetrate into the plant. Thus, tricylazole is a nonfungicidal compound which acts through interference with the pathogenic process. Other compounds such as probenazole increase host resistance in plants challenged by pathogens (*19*). Dichloroisonicotinic acid is a promising new experimental compound of this type which is nonfungitoxic but increases host resistance to both fungal and bacterial pathogens (*20*). Future directions for chemical disease control will undoubtedly include more compounds that interfere with the disease process without direct toxicity to the pathogen. Progress in this area, however, is dependent on an increase in fundamental knowledge of fungal pathogenicity and host/parasite interactions.

Human Exposure

As a general rule, fungicides and their residues have a low mammalian toxicity and are rarely the cause of acute intoxication. Some sensitive individuals develop contact allergies following exposure to fungicides, but generally fungicides do not present a reentry problem for agricultural workers (*21*). Data from the state of California, which monitors accidents and illnesses occurring in agricultural production, indicate that in 1987 1,507 illness cases were related to pesticide exposure during occupational duties (*3*). Of this number, a total of 234 cases were linked to fungicide use with sulfur exposure accounting for 44 percent of the cases. Thus, if the cases attributed to sulfur were removed (since it is also used as an insecticide and acaricide), 130 cases remain, representing 8.6 percent of the occupationally related pesticide illnesses. Studies have shown the highest likelihood for pesticide exposure occurs during the mixing and loading operations prior to application (*21*). This potential risk as well as many of the occupationally related exposures can be reduced or eliminated by the use of protective gear and adjustments in working practices. Success in this area must rely on educational programs for agricultural workers.

The primary concerns about human exposure to fungicides have focused on chronic toxicity, carcinogenicity in particular. Since very little, if any, reliable data exist on human carcinogenesis resulting from pesticide exposure, data from animal studies are used to estimate the likelihood of cancer occurrence. If one examines the background for the concerns related to fungicides, two factors become evident:

1. Low acute toxicity means that large quantities of chemicals can be fed to animals (usually rats and mice) without killing them. In the United States a range of pesticide doses is used that is increased to the Maximum Tolerated Dose (MTD), the

highest dose that results in toxic effects during chronic exposure without causing death and does not decrease body weight by more than 10 percent.

2. When large quantities of fungicides are fed to animals, the chemicals may saturate or inhibit enzyme systems that would rapidly metabolize the chemical or show little effect at doses encountered in reality. The extrapolation procedures from effects on rodents at high doses to humans exposed to low doses is highly controversial. The fungicides that are multisite inhibitors are particularly likely to be vulnerable in this testing protocol. Others could also face difficulties on this same premise depending upon cellular sites of action and whether these or similar sites are present in mammalian systems.

The risk of cancer extrapolated from long-term rodent feeding studies coupled with maximum potential pesticide residue levels (tolerances) in food was examined recently in a study conducted by the Board on Agriculture of the National Research Council (NRC) (*23*). The resulting publication, "Regulating Pesticides in Food: the Delaney Paradox," presented conclusions showing rather high cancer risks from pesticides in foods, and in particular from fungicides. The total hypothetical cancer risk from pesticides in food was presented as 5,800 per million with 3,500 (60 percent) of the total attributed to fungicides. The unrealistic information was reported by the press in rather alarming language, and the public has been unduly concerned ever since about food safety in relation to pesticide residues. An examination of data from a number of residue monitoring programs reveals that fungicides applied in the production of food seldom result in detectable residues in food (*21*). When they are found, they are usually orders of magnitude less than the allowable residue levels (tolerances). When Archibald and Winter (*22*) used actual residue and incidence data from the Los Angeles FDA laboratory to recalculate the cancer risk resulting from fungicide residues on tomatoes, they determined it to be 0.24 per million as opposed to 823 per million indicated in the NRC study (*23*). A factor completely overlooked when the study was presented to the public was the important role fungicides play in preventing food contamination with toxic fungal products (mycotoxins) and plant defense chemicals (phytoalexins). These hazards may exceed any small risk involved in the use of fungicides to control this contamination.

The question still remains concerning the possible effects of chronic exposure to fungicides. There are no published scientific data that indicate whether or not problems exist at the low levels that are likely to be actually encountered. However, there is no question that under the current guidelines for cancer assessment, the new fungicides are less likely to encounter problems related to carcinogenic risk estimates.

Environmental Considerations

The fate and effects of fungicides in the environment may be examined at three levels. First there is the movement off the immediate site of application; second is fate in the immediate environment; and third is the plant (site) itself.

In examining the effects of movement off the site of application, one finds that the ultimate fates of many fungicides have not been determined. Because of their low acute non-target toxicity, they have not been as actively sought in environmental monitoring programs as other classes of pesticides. Also, the amounts of fungicides used in the United States (U.S.) are much less than insecticides and herbicides; and fungicide residues are rarely found in random sampling of soil, water, and air. Generally fungicides have short half-lives, but these can very considerably depending on a number of factors such as chemical structure, and climatic and edaphic factors.

Since the older organic fungicides generally have low volatility, fungicide residues are not likely to be detected in air samples other than immediately after spray application (*21*). Monitoring surveys for six pesticides in the air of several California communities detected small amounts of captan residues consistent with patterns of

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

use in the vicinity (*24*). While the newer fungicides demonastrate more volitility, the rates applied have given no indication of off site non-target effects.

Fungicide residues are rarely found in surface water, and there have only been a very limited number of detections in ground water in the U.S. Direct soil application and/or incorporation are important factors contributing to the potential for ground water contamination. Oregon State University surveyed state agencies responsible for ground water protection in the fifty States to determine the extent of ground water contamination by pesticide residues used in agriculture. Of the 67 pesticide residues identified in 6,032 positive out of 122,881 wells tested, only two were fungicides; they were PCNB and chlorothalonil (*25*).

When the fate of a fungicide in the immediate environment is considered, the soil in the vicinity of the plants treated is the area of concern. Half-lives of fungicide residues in soil are highly dependent on the rate of application and local conditions (*21*). For example, captan residues may remain in the soil from one day to several months depending on soil type, temperature, and moisture content (*26*). Formulation may also be a factor in persistence and degradation. Undoubtly this issue will be more closely monitored in future developmental investigations.

Microbial degradation may be the single most important factor in preventing buildup of a fungicide. In certain cases soil microorganisms degrade specific pesticide residues so rapidly that their effectiveness is reduced or eliminated (*27*). This process is know as "enhanced" or "accelerated" microbial degradation and is the result of a process known as "selective enrichment" in which the microbial population is altered by the presence of a certain pesticide acting as a nutrient. The dicarboximide fungicides iprodione and vinclozolin have had efficacy problems related to enhanced degradation (*28*). Additional research is needed in the area to determine the many factors which influence this process. The first essential strategy for managing the problem is using a maximum rotation of chemicals with varying structures and modes of action.

In some situations there is concern that some fungicides persist too long. The widespread use of the triazole fungicides has raised concern about effects of these fungicide residues on subsequent crops (*21*). In some situations the half life of triazole fungicides in soil has been shown to be greater than one year (*29*). Although these fungicides are not usually applied directly to the soil, they reach it during foliar application, rain wash off, and leaf drop. Such persistence creates concerns not only for effects on mycorrhizal associations in future crops but also for increased chances of the development of fungal resistance to the fungicide as a result of the prolonged selection pressure.

The soil ecosystem is extremely complex, and the introduction of crop systems is disruptive in itself. The addition of pesticides can have positive and negative effects. Fungicide residues may modify the degradation rates of other pesticides. For example, fungicide residues can increase the half-life of the insecticide carbofuran by 1.2 to 3 times compared to the carbofuran half-life in isolation (*30*). This situation indicates the fungicides have adversely affected the soil fungal population that would normally degrade the insecticide. This can be a valuable factor when some persistence is necessary to accomplish the pest control the insecticide is intended to deliver.

Beyond effects on microorganisms, some fungicides have been shown to affect other components of the soil ecosystem. A great deal of concern has been expressed over the fact that use of the benzimidazoles has temporarily reduced earthworm populations under some conditions (*31*). In apple orchards where earthworms fed on benzimidazole-treated plant debris, populations were reduced. In situations where earthworms are critical, excessive use of benzimidazoles should be avoided.

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

Fungicides that are applied to the soil, or come in contact with it following above ground application, may be toxic to microorganisms other than pathogenic fungi (*32*). Alterations of the ecosystem in the rhizosphere can have beneficial as well as antagonistic effects in agricultural production. Generally the assumption is made that use of a particular pesticide enhances production. The indirect and ecological effects of a given pesticide are difficult to assess (*33*). However, if optimal production under a specific crop management regime in a specific location is sought, the effects of pesticides on the soil ecosystem should be considered. There are various indicator processes in the soil that can be measured such as effects on respiration in soil cores, effects on invertebrate and microbial biomass, and effects on breakdown of organic matter (*34*). If such studies are undertaken, consideration should be given to the soil, hydrological, and climatic conditions in the area under investigation (*35*).

Fungicide applications to plant surfaces may alter the microorganisms present (*28*), cause phytotoxicity or growth regulatory effects, or stimulate the plant defense system as discussed previously. Alteration of microorganisms on a plant surface may be beneficial or antagonistic to maximum production, and studies of pesticide effects in this regard can be important to integrated control strategies (*36*).

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

Industrial Perspective

The discovery of effective plant disease control agents is an increasing challenge as we approach the 21st century. Not only must new materials meet customer expectations of cost and efficacy at very low use rates with minimal resistance risk, but these materials must meet new standards of environmental safety. Industry is currently taking a proactive approach to discover and develop new products with minimum environmental impact and an improved toxicological profile. To achieve this goal several factors are considered in the discovery process:

Low Use Rates. A low use rate is probably the key factor in developing better and safer materials for controlling agricultural plant pathogens. As industry has succeeded in discovering more efficacious materials which act at specific targets within plant pathogens, much less compound is now required to control these pathogens. As adverse effects are almost inevitably dose dependent, the more selective the material the fewer indirect problems result.

Mode of Action. Low use rates are also linked to specific modes of action. The more potent and selective a compound is toward a specific mode of action, the less material is needed to control the pathogen of interest. It is now a standard practice in industrial discovery research laboratories to use specific enzyme assays in parallel with whole plant disease control tests to maximize the potency of new candidate plant disease control agents. In addition to focusing on improved potency, mode of action is a factor which is used to maximize selectivity and safety. Clearly the exploitation of biochemical pathways and specific enzyme targets which are not present in non-target species provides one level of safety. An example of such a mechanism is found with the compound tricyclazole that inhibits the enzyme 1,3,8 -trihydroxynapthalene reductase of the polyketide pathway which produces a form of melanin which is not found in mammals (*37*). This target site thus meets a very desirable feature of being specific for the pest we are trying to control. Subcellullar targets need not always be unique to the fungus, as many successful plant disease control agents such as the benzimidazoles, azoles, carboxamides, and phenylamides as described earlier in this chapter act on targets which are present in fungal pathogens and non-target species and yet possess enough selectively to be safely used and widely accepted. Although it is desirable to exploit targets unique to fungal pathogens, this is not always possible. The ability to directly measure the inhibitory effect at the target site gives us the ability to develop methods to maximize the activity on the fungal target and avoid the non-target.

Environmental and Toxicological Factors. The following factors all specifically relate to critical features required in registration of new plant disease control agents. Unless compounds are related to existing products, prediction of environmental stability and toxicological profile is unreliable. Therefore, firsthand studies must be performed; this occurs only if sufficient interest in a particular compound warrants the initiation of environmental and toxicological studies. It is during this stage of development that adverse effects are typically identified which may activate efforts to determine the necessary modification of compounds to avoid the problem.

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

Stability in the Soil and Water. The stability of a compound in the environment is another area that industry examines during compound development. Although it is impractical to evaluate this parameter for all antifungal leads, the stability of a compound in soil and water is often examined as candidates progress toward development, especially if there is a reason to suggest that this may ultimately be a limiting factor. The stringent regulations for compound half-life in Japan and the European Community justify this research. Depending on the interest of the chemical lead, a wide variety of projects can be initiated to optimize residual disease control while identifying candidates which do not accumulate in the environment. Features such as the physical parameters affecting stability in soil and water such as soil type, pH, temperature and water hardness and biotic factors such as microbial degradation may be examined. It is important to determine if the response of compounds concerning these parameters is common to all compounds in a series or if differences exist which are unique to specific compounds. It is the latter which allows us to use an information based approach to minimizing non-target effects.

Toxicity to Non-Target Species. As with stability, adverse toxicity to non-target species is a parameter which can be actively investigated if there is sufficient interest in a particular lead, or lead area of chemistry. Again, as with stability in the environment, the difficulty in identifying this problem is the fact that toxicity problems cannot be predicted and only arise during safety evaluations of advanced compounds. Once a problem has been identified and characterized, only then can tests be designed to discover compounds to specifically avoid a particular toxicity problem. Experiments to resolve these specific problems are not nearly as elaborate as those required for registration purposes, but can direct chemistry to avoid the toxicity problem. An example of this is fish toxicity which is an extremely important non-target species concern for compounds which control rice pathogens.

Similarity to Known Hazardous Materials. Very early in the discovery process many compounds are routinely screened that result in more weakly active compounds than most companies can pursue. In an effort to minimize chances of having problems later, some compounds which are related to older well known problem chemistries, such as chlorinated hydrocarbons and heavy metal containing compounds are avoided at this stage

Mutagenicity Parameters. The Ames assay has provided opportunities to examine mutagenicity problems with minimal time and effort. Although not always a perfect indication of the actual mutagenicity risk, it is still a tool for industry to employ and avoid potential problems.

Minimizing non-target effects of plant disease control agents is possible in today's research environment when industry approaches it as a problem that can be

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

solved rather than avoided. By identifying the specific concerns that need to be resolved, a research strategy can be established to solve these problems early in a rational process rather than reacting to them late in the development process when the investment of time and resources is at its height.

Literature Cited

1. McCallan, S.E.A. In *Fungicides*; Torgeson, D.C., Ed.; Academic Press: New York, NY, 1967. Vol. 1: pp. 1-37.
2. Ragsdale, N.N.; Sisler, H.D. In *Handbook of Pest Management in Agriculture, 2nd Ed*; Pimental, D., Ed.; CRC Press: Boca Raton, FL, 1991. Vol. 2; pp. 461-496.
3. Edminston, S.; Richmond, D. *California Summary of Illness and Injury Reported by Physicians as Potentially Related to Pesticides*, CDFA Division of Pest Management, Environmental Protection and Worker Safety; Sacramento, CA, 1988.
4. Tisdale, W.H.; Williams, I. *Disinfectant and Fungicide,* U.S. Patent 1, 972, 961, 1934.
5. Crowdy, S.H. In *Systemic Fungicides*; Marsh, R. W., Ed.; Longman: London, 1972; p. 113.
6. Delp, C.J. In *Modern Selective Fungicides*; Lyr, H., Ed.; Longman Group Ltd./VEB Gustav Fischer Verlag: London/Jena, 1987; pp. 234-257.
7. Edgington, L.V. *Ann. Rev. Phytopathol.* **1981**, *19*, 107-124.
8. Scheinpflug, H. In *Fungicide Chemistry: Advances and Practical Applications*; Green, M. B.; Spilker, D. A., Eds.; American Chemical Society: Washington, DC, 1986; pp. 73-88.
9. Sisler, H.D.; Ragsdale, N.N. In *Agricultural Chemicals of the Future,* Hilton, J.L., Ed.; Rowman and Allanhold: Totowa, NJ, 1985; pp. 175-188.
10. Somerville, L. *Xenobioties* **1986**, *16*, 1017-1030.
11. Buchenauer, H. *Pestic. Sci.* **1975**, *6*, 525-535.
12. Mathre, D.E. *Pestic. Biochem. Physiol.* **1971**, *2*, 216-224.
13. Fenn, M.; Coffey, M.D. *Calif. Avocado Soc. Yrbk.* **1987**, *17*, 241-249.
14. Sisler, H.D.; Ragsdale, N.N. In *Modern Selective Fungicides*; Lyr, H., Ed.; Longman Group Ltd./VEB Gustav Fischer Verlag: London/Jena, 1987; pp. 337-353.
15. Griffith, J.M.; Davis, A.J.; Grant, B.R. In *Target Sites of Fungicide Action,* Koller, W. Ed.; CRC Press: Ann Arbor 1992 pp. 70-100.
16. Davidse, L.C. In *Modern Selective Fungicides,* Lyr. H., Ed.; Longman Group Ltd./VEB Gustav Fischer Verlag: London/Jena, 1987; pp. 259-273.
17. Edlich, W.; Lyr, H. In *Modern Selective Fungicides,* Lyr, H., Ed.; Longman Group Ltd./VEB Gustav Fischer Verlag: London/Jena, 1987, pp. 107-118.
18. Pommer, E.H.; Lorenz, G. In *Modern Selective Fungicides*, Lyr. H., Ed.; Longman Group Ltd./VEB Gustav Fischer Verlag: London/Jena, 1987; pp. 91-106.
19. Sekizawa, Y.; Mase, S. *J. Pestic. Sci.* **1981** *6*, 91-94.
20. Metraux, J. P.; Goy, P. A.; Staub, T.; Speich, J.; Steinemann, A.; Ryals, J.; Ward, E. In *Advances in Molecular Genetics of Plant-Microbe Interact.* Hennecke, H.; Verma, D., Eds.; Kluwer Academic: Dordrecht, 1991, Vol. 1; pp. 432-439.
21. *Health and Environmental Factors Associated with Agricultural use of Fungicides*; Ragsdale, N.N.; Hylin, J.W.; Sisler, H.D.; Witt, J.M. NAPIAP Fungicide Assessment Report; USDA, Washington, DC, 1991.

22. Archibald, S.O.; Winter, C.K. In *Chemicals in the Human Food Chain*; Winter, C.K.; Seiber, J.N.; Nuckton, C.F., Eds.; Van Nostrand and Reinhold: New York, 1990; pp. 1-50.
23. *Regulating pesticides in Food: the Delaney Paradox.* National Academy Press: Washington, DC, 1987.
24. Seiber, J.N. In *Pesticides: Minimizing the Risks*, Ragsdale, N.N.; Kuhr, R.J., Eds.; American Chemical Society: Washington DC, 1987, pp. 88-105.
25. Parsons, D.W.; Witt, J.M. *Pesticides in Groundwater in the United States of America: A Report of a 1988 Survey of State Lead Agencies,* Oregon State University, Extension Service: Corvallis, OR, 1988.
26. Buyanovsky, G.A.; Pieczonka, G.J.; Wagner, G.H.; Fairchild, M.L. *Bull. Environ. Contam. Toxicol.* **1988**, *40*, 689-695.
27. Reed, J.P.; Kremer, R.J.; Keaster, A.J. *Bull. Environ. Contam. Toxicol.* **1987**, *39*, 776-782.
28. Suett. D.L. In *Brighton Crop Protection Conference, pests and Diseases - 1990*, Lavenham Press Ltd.; Lavenham, UK, 1990; Vol. 3; pp. 897-906.
29. Patil, S. G.; Nicholls, P. H.; Chamberlain K.; Briggs G.G.; Bromilow R. H., *Pestic. Sci.* **1988** *22*, 333-342.
30. Pussemier, L.J. *Environ. Sci. Health* **1988,** *B 23*, 193-209.
31. Stringer, A.; Lyons, C.H. *Pestic. Sci.* **1974,** *5,* 189-196.
32. Vyas, S.C. *Nontarget Effects of Agricultural Fungicides*; CRC Press; Boca Raton, FL, 1988.
33. Graham-Bryce, I.J. In *Brighton Crop Protection Conference, pests and Diseases - 1988*; Lavenham Press Ltd.: Lavenham, UK, 1988; Vol. 2; pp. 571-580.
34. Edwards, C.A. In *Brighton Crop Protection Conference, pests and Diseases - 1988*; Lavenham Press Ltd.: Lavenham, UK, 1988; Vol. 2; pp. 739-746.
35. Hollis, J.M.; Carter, A.D. In *Brighton Crop Protection Conference, Pests and Diseases - 1990*; Lavenham Press Ltd.: Lavenham, UK, 1990; Vol. 3; pp. 1005-1010.
36. De Cal, A.; Melgarejo, P. In *Brighton Crop Protection Conference, Pests and Diseases - 1988*; Lavenham Press Ltd.: Lavenham, UK, 1988; Vol. 2,; pp. 733-738.
37. Woloshuk, C.P.; Sisler, H.D.; Tokousbalides, M.C.; Dutkey, S.D., *Pestic. Biochem. Physiol.* **1980** *14*, 256-264.

RECEIVED September 9, 1992

Downloaded by UNIV OF GUELPH LIBRARY on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ch024

Author Index

Abell, L. M., 16
Addor, R. W., 219
Bjostad, Louis B., 199
Black, B. C., 219
Carlson, P. S., 87
Carlton, Bruce C., 258
Casida, John E., 126
Chitwood, David J., 300
Christy, A. L., 87
Cranshaw, Whitney S., 199
Diehl, R. E., 219
Donn, G., 38
Duke, Stephen O., 1,62,110
Eckes, P., 38
Evans, S. L., 79
Fisher, M. H., 169
Fravel, D. R., 323
Furch, J. A., 219
Gressel, Jonathan, 48
Hammock, Bruce D., 281
Hatakoshi, M., 144
Hawkinson, Jon E., 126
Henegar, K. E., 219
Henry, M. J., 332
Herbst, K. A., 87
Hibbard, Bruce E., 199
Hirano, M., 144
Jenkins, Johnie N., 267
Kamhi, V. M., 219
Kostka, S. J., 87
Kremer, K. A., 219
Kuhn, D. G., 219
Lowen, G. T., 219
Lydon, John, 110
Maeda, Susumu, 281
Menn, Julius J., 1
Miller, R. Vincent, 101
Miller, T. P., 219
Miyamoto, J., 144
Morton, H. V., 316
Müllner, H., 38
Mullen, J. P., 87
Mullins, J. W., 183
Nandihalli, Ujjana B., 62
Nyfeler, R., 316
Piek, T., 233
Plimmer, Jack R., 1
Ragsdale, N. N., 332
Rendina, A. R., 16
Roberts, D. P., 323
Sands, David C., 101
Savage, S. D., 79
Schloss, J. V., 16
Sisler, H. D., 332
Takimoto, Y., 144
Treacy, M. F., 219
Vaughn, James L., 239
Zorner, P. S., 79

Affiliation Index

ABC Laboratories, 1
American Cyanamid Company, 219
Ciba-Geigy Corporation, 316
Ciba-Geigy, Ltd., 316
Colorado State University, 199
Crop Genetics International, 87
Delta State University, 62
DowElanco, 332
E. I. du Pont de Nemours and Company, 16
Ecogen, Inc., 258
Hoechst AG, 38
Merck Sharp and Dohme Research Laboratories, 169
Miles Inc., 183
Montana State University, 101
Mycogen Corporation, 79
Sumitomo Chemical Company, Ltd., 144
U.S. Department of Agriculture, 1,62,110,239,267,300,323,332
University of Amsterdam, 233
University of California—Berkeley, 126
University of California—Davis, 281
University of Kansas School of Pharmacy, 16
University of Maryland—College Park, 332
Weizmann Institute of Science, 48

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix001

In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.

Subject Index

A

AAL toxin
  phytotoxicity, 118
  structure, 116*f*,118
Abamectin
  activity against mites and insects, 171*t*
  application, 169
  structure, 171,172*f*
AC 303,630
  properties and structure, 229,231*f*
  structure–activity relationship, 228,229*t*
  toxicities, 229,230*f*
  use in integrated pest management systems, 229,231*f*
Acetolactate synthase, inhibition, 30*f*,31
Acetyl coenzyme A carboxylase, inhibition, 29,30*f*
Acetylenes, nematicidal activity and structures, 303,304–305
Acifluorfen, structure, 71*f*
Acivicin
  structure, 24*f*
  use as enzyme inhibitor, 24–25
Aerobic soil, metabolism of pyriproxyfen, 160
Affinity labels
  structure, 24*f*
  use as enzyme inhibitors, 24–25
Affinity probes, noncompetitive blocker site on γ-aminobutyric acid receptors in mammals, 133
Agricultural antibiotics, use of natural products, 317,318*t*
Agricultural efficiency, need, 38
Agriculture, concern about environmental consequences, 39
Agrobacterium tumefaciens, production of transgenic cotton plants, 270
Agrochemicals, function, 38
Alachlor, structure, 22*f*
Alantolactone
  nematicidal activity, 311
  structure, 310*f*,311
Alkaloids
  nematicidal activity, 301,303
  phytotoxicity, 114
  structures, 301,302*f*
Allelochemicals
  definition, 111
  phytotoxicity, 111–112
Allelopathic compounds, development, 10–11
Allidochlor, structure, 22*f*
Allygrin
  nematicidal activity, 311
  structure, 310*f*,311
Allyl isothiocyanate
  nematicidal activity, 308
  structure, 308,310*f*
Alternatives to synthetic chemical pesticides, reasons for development, 258
Amidases, inhibition by synergists, 56
Aminobenzotriazole, monooxygenase inhibition, 52–53,54*t*
γ-Aminobutyric acid, functions, 126–129
γ-Aminobutyric acid induced chloride-36 flux, enhancers and inhibitors, 127
γ-Aminobutyric acid receptor
  future as insecticide targets, 140
  structure, 127–128*f*
L-(α-*S*,5*S*)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, *See* Acivicin
cis-2-Amino-1-hydroxycyclobutane-1-acetic acid
  phytotoxicity, 115
  structure, 115,116*f*
δ-Aminolevulinic acid
  as porphyric herbicide, 64–65,67–68
  phytotoxicity, 112
  structure, 112,113*f*
δ-Aminolevulinic acid dehydratase, as herbicide target site, inhibition, 73*f*
Ancymidol, monooxygenase inhibition, 52–53,54*t*
Anisomycin, phytotoxicity, 115,117
Antibiotics, use in biocontrol, 326–327
Antifeedants, use of semiochemicals, 208
Antisense RNA, enzyme inhibition, 20–21
Apple codling moth, control with baculoviruses, 248
Aquatic environment
  ecological effects of diflubenzuron, 153–154
  ecological effects of methoprene, 155
Aquatic organisms, bioaccumulation and metabolism of pyriproxyfen, 163,164*f*

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Arthropod-derived neurotoxic insecticides, effects, 234–237
2-Aryl-3-cyano-4,5-dihalopyrroles and 2-Aryl-3-nitro-4,5-dihalopyrroles
  insecticidal activity, 223,224–225*f*
  synthesis, 220,221*f*
2-Aryl-5-(trifluoromethyl)pyrrole derivatives
  insecticidal activity, 223,225*t*
  synthesis, 220,222*f*,224*f*
Ascorbate, oxidant detoxification pathway suppression, 55
Asparagusic acid
  nematicidal activity, 308,311
  structure, 310*f*,311
Attractants, history of use, 207
Attracticides, use of semiochemicals, 207–208
Auxotrophy, zip code system, 106–107,108*t*
Avermectin(s)
  advantages, 136
  applications, 169,171
  discovery, 169
  pharmacological effects, 176,179*t*
  structures, 138,169,170*f*
  synthetic program to discover compounds with increased insecticidal activity, 173–182
  types of action, 136,138
Avermectin B1, structure, 171,174*f*
Avermectin-binding protein
  affinity probe, 176,180*f*
  isolation, 180,181*f*,182
Avermectin binding site in mammals, insects, and nematodes
  binding affinity, 138,139*f*
  identification of binding protein, 138,140
Avermectin spiroketal, cleavage, 173,175*f*
Azadirachtin
  nematicidal activity, 311
  structure, 310*f*,311
Azidoavermectin
  activity, 180
  binding affinity, 180,181*f*,182

B

Bacillus popilliae, production process, 259
Bacillus thuringiensis
  complexity, 260–262
  developments, 259–260
Bacillus thuringiensis—Continued
  improvement methods, 262–266
  insecticidal crystal proteins, 271–272
Bacillus thuringiensis gene use in transgenic cotton to control lepidopterous insects
  biotechnology, 269–270
  development of endotoxin for insect control, 271
  experimental results, 276–278
  insecticidal crystal proteins present in *Bacillus thuringiensis*, 271–272
  limitations, 273
  management strategies, 274–276
  mode of action of insect control proteins, 272–273
  recombinant DNA technology, 269
  resistance mechanisms, 273–274
  systems for producing transgenic plants, 270
Bacillus thuringiensis insect control proteins, mode of action, 272–273
Bacillus thuringiensis toxin
  insecticidal activity effect, 289–290
  use in construction of recombinant baculoviruses, 287
Bacteria-based bioherbicides
  examples, 82–83,92
  greenhouse screens, 94,95*f*
  preparation of bacteria–herbicide tank mixes, 94
Baculoviridae, applications, 244
Baculovirus(es)
  cattle application, 248–249
  characterization, 244–245
  cole crop application, 246,248
  development, 6
  expression vectors, 282,284
  forest application, 249–250
  future prospects, 293–294
  gene structure, 282
  improvements in biological activity, 290–291,292*f*,293
  orchard crop application, 248
  polyhedral-shaped inclusion bodies, 282,283*f*
  potentially controllable agricultural pests, 246,247*t*
  replication, 282
  safety, 293
  soybean application, 249

Baculovirus genome, polypeptide or peptide genes useful for insertion, 285–287
Barbiturates, binding sites, 128*f*,129
BAS110, monooxygenase inhibition, 52–53,54*t*
BAS111, monooxygenase inhibition, 52–53,54*t*
Bees, ecological effects of diflubenzuron, 154
Behavioral pest control, use of semiochemicals, 205–208
Benomyl, use as fungicide, 334
Benzodiazepines, binding sites, 128*f*,129
Bialaphos
  phytotoxicity, 115
  structure, 115,116*f*
Bialaphos resistance gene, use in gene transfer, 44
Binding kinetics, noncompetitive blocker site on γ-aminobutyric acid receptors in mammals, 133,135*f*
Bioavailability, pyriproxyfen, 163,165*t*
Biocontrol
  antibiotics, 326–327
  elements, 323–324
  enzymes, 327–328
  justification, 101–102
  nutrient competition, 328,329*t*
Biocontrol agents
  advantages and disadvantages, 87–88
  development, 6
  history of use to control pests, 87
  introduction, 325–326
  replacement for chemical pesticides, 323
  synergism with chemical herbicides, 87
  synergizing, 57
Biocontrol of weeds, development, 11
Biocontrol strategies
  destruction of pathogen inoculum, 324
  protection of rhizosphere or spermosphere, 324
  retarding pathogen growth through soil, 324–325
Bioherbicides
  advantages, 79–80
  bacteria, 82–83
  bacteria based, *See* Bacteria-based bioherbicides
  correct target choice for commercialization, 83–84
Bioherbicides—*Continued*
  current scientific effort, 80
  factors important for commercialization, 81
  fermentation effect on commercialization, 84–85
  fungal based, *See* Fungal-based bioherbicides
  importance of recognition of biological principals for commercialization, 81–82
  stabilization effect on commercialization, 84–85
  technical barriers for commercialization, 84
Bioinsecticides based on *Bacillus thuringiensis*, development, 263–266
Biological containment systems, advantages, 102
Biological herbicides, *See* Bioherbicides
Biological materials
  factors affecting growth, 2
  market, 1
Biological pesticides, lack of acceptance in pest control marketplace, 259
Biological principals, recognition for commercialization of bioherbicides, 81–82
Biopesticides, market value, 10
Biotechnological methods, impact on pest management, 10–11
Biotechnological products, market value, 10
Biotechnology, plant science effect, 269–270
Bocconine
  nematicidal activity, 303
  structure, 302*f*,303
Broad host range of plant pathogens, examples, 102
Brown plant hopper, control with resistant gene, 268
Bt toxin, *See Bacillus thuringiensis* toxin
Buprofezin
  insecticidal activity, 147*t*
  structure, 145*f*
5-(3-Buten-1-yl)-2,2′-bithienyl
  nematicidal activity, 301
  structure, 301*f*
Butyric acid
  nematicidal activity, 306
  structure, 306,307*f*

C

C_{24}–C_{25} avermectins, Wittig approach for synthesis, 173,177*f*

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Cabbage looper
  Bacillus thuringiensis gene effect, 278
  control with baculoviruses, 246
  control with virus, 240
Cabbageworm, control with baculoviruses, 246
Cancellation of pesticides, process, 4
Carboxamides, uses as fungicides, 334
Carboxybiotin, structure, 29,31*f*
Carotenes, oxidant detoxification pathway suppression, 55
Centaurea maculosa, *See* Spotted knapweed
CGA–41396
  structure, 321*f*
  systemic acquired resistance, 321
CGA–41397
  structure, 321*f*
  systemic acquired resistance, 321
CGA–173506
  activity, 319,320*t*
  structure, 319*f*
Chaparrinone
  nematicidal activity, 311
  structure, 310*f*,311
Chelerythrine
  nematicidal activity, 303
  structure, 302*f*,303
Chemical herbicides, synergism with weed biocontrol agents, 88–99
Chemical pest controls, nonconventional, *See* Nonconventional pest controls
Chemical pesticides
  interest in alternatives, 323
  public perception, 258
Chemical validation, targets for herbicide design, 18–19
Chitin, biosynthetic pathway, 148,149*f*
Chitin synthesis inhibitors
  insecticidal activity, 146–147*t*
  mode of action, 147–148,149*f*
  structures, 144,145*f*,146
Chlorfluazuron
  insecticidal activity, 146*t*
  structure, 145*f*,146
Chloroacetamide herbicides
  function, 21–22
  structures, 21,22*f*
2-Chloro-*N*,*N*-diallyl-acetamide, *See* Allidochlor
Chlorophyll content, measure of herbicide phytotoxicity, 62
Chlorothalonil, use as fungicide, 333
Chlorsulfuron, structure, 29,31*f*
Cholinergic nerve endings, irreversible depletion by kinins, 234–235,236*f*
Chorismate synthase, inhibitors, 19
1,8-Cineole
  nematicidal activity, 306
  phytotoxicity, 112
  structure, 112,113*f*,306,307*f*
Cinmethylin
  phytotoxicity, 112
  structure, 112,113*f*
Citral
  nematicidal activity, 306
  structure, 306,307*f*
Clethodim, structure, 29,31*f*
Cole crops, use of baculoviruses for pest control, 246,248
Colletotrichin, phytotoxicity and structure, 116*f*,118
Commercialization, influencing factors for bioherbicides, 81–85
Condor, development by nonrecombinant approaches, 263
Containment and concentration of pests, use of semiochemicals, 206
Control of pests, *See* Pest Control
Conventional plant breeding
  description, 269
  discovery of genes for resistance, 269
Copper chelator–synergists, Halliwell–Asada pathway suppression, 55–56
Corn
  rate of glufosinate required for weed control for tolerant crops, 45*t*,46
  weed control systems, 40*t*
Corn rootworm complex, management approaches, 8
Cornexistin, phytotoxicity and structure, 116*f*,117
Cotton, use of baculoviruses for pest control, 248–249
Cotton bollworm, *Bacillus thuringiensis* gene effect, 278
Cotton leaf perforator, *Bacillus thuringiensis* gene effect, 278
Cotton plants, transgenic, *See* Transgenic cotton plants

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Coumestrol, nematicidal activity and structure, 308,309*f*
Crop protection chemicals
  function, 1
  market value, 1
Crop resistance, engineering, 38–46
Crop tolerance, herbicides, 7
Cultivars, pest resistant, *See* Pest-resistant cultivars
Cutlass, development by nonrecombinant approaches, 263
Cytoplasmic polyhedrosis viruses, description, 241

D

2-*trans*,8-*cis*-Deca-2,8-diene-4,6-diynoate, nematicidal activity and structure, 303,304*f*
cis-Dehydromatricaria and *trans*-dehydromatricaria
  nematicidal activity, 303,306
  structures, 303,304–305*f*
cis-Dehydromatricaria ester, nematicidal activity and structure, 303,304*f*
Dehydroquinate synthase, inhibitors, 19
Density of pests, estimation with semiochemicals, 202
Diamondback moth, control with baculoviruses, 246,248
Di-*N*-butyl succinate, nematicidal activity and structure, 306,307*f*
Dicarboximides, use as fungicides, 335
3-(2,4-Dichlorophenoxy)-1-propyne, monooxygenase inhibition, 52–53,54*t*
Diclofop, structure, 29,31*f*
Diflubenzuron
  activity, 145–148
  ecological effects in environment, 153–154
4-(2,2-Difluoro-1,3-benzodioxol-4-yl)-pyrrole-3-carbonitrile, activity and structure, 319,320*t*
2,3-Dihydro-2-hydroxy-3-methylene-6-methyl-benzofuran, nematicidal activity and structure, 310*f*,311
Dihydroquinone sorgoleone, phytotoxicity and structure, 112,113*f*
Dihydroxy acid dehydratase, inhibitors, 19
1,4-Dihydroxy-1,4-benzoxazin-3-one, phytotoxicity and structure, 112,113*f*
Dioxapyrrolomycin, insecticidal activity and structure, 219,221*f*
Diphenyl ether analogues, quantitative structure–activity relationships, 72
Diphenyl ether herbicides, mode of action, 68,69*f*
Dipropyl thiosulfinate, nematicidal activity and structure, 310*f*,311
2,2′-Dipyridyl, phytotoxicity and structure, 112,113*f*
Disease control, use of microbial metabolite derivatives and plant defenses, 316–321
Dithianes, noncompetitive blockers of γ-aminobutyric acid receptor binding site, 131,132*t*
Diuretic hormone, insecticidal activity effect, 289
Doramectin
  application, 169,171
  structure, 171,172*f*
Douglas fir tussock moth, control with baculoviruses, 249
DU–19111, insecticidal activity and structure, 144,145*f*,146

E

Ecological effects
  diflubenzuron, 153–154
  methoprene, 154–157
  pyriproxyfen, 157,158–159*f*,160
Edifenphos, amidase inhibition, 56
Efficacy, pest control approaches, 6
Egyptian cotton leafworm, control with baculoviruses, 248–249
EL–499, structure, 227
Emamectin
  foliar ingestion activity against insect larvae and adult spider mites and aphids, 173*t*
  structure, 173,175*f*
Engineered baculoviruses, development, 6
Enhancer-type modulators, mechanism of action, 66–67
Environment, degradation of insect growth regulators, 160–165
Environmental concerns
  fungicides, 336–338
  pesticide use, 3
Environmental fate studies, 5

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

American Chemical
Society Library
1155 16th St. N. W.
Washington, D. C. 20036

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Environmental safety, challenge of pest control, 1–12
Enzyme(s)
  selection as target for herbicide design, 17–18
  use in biocontrol, 327–328
  use in construction of recombinant baculoviruses, 285
Enzyme inhibitor(s), use in construction of recombinant baculoviruses, 285
Enzyme inhibitor design
  affinity labels, 24*f*,25
  extraneous site inhibitors, 29–30,31*f*,32
  ground-state analogues, 22,23*f*,24
  group-specific reagents, 21,22*f*
  reaction intermediate analogues, 25–30
  suicide substrates, 25,26*f*
4′-Epiaminoavermectins, synthesis, 173,174*f*
9,10-Epoxyheptadec-16-en-4,6-diyn-8-ol, nematicidal activity and structure, 303,304*f*
Esterases, inhibition by synergists, 56
Eugenol, nematicidal activity and structure, 306,307*f*
European pine sawfly, control with baculoviruses, 249
European spruce sawfly, control with virus, 240
Exotic pests, monitoring with semiochemicals, 201
Expression vectors, baculoviruses, 282,284
Extraneous site inhibitors, mechanism, 29–32

F

Fatty acids, nematicidal activity and structures, 306,307*f*
Federal Environmental Pest Control Act, function, 4–5
Federal Insecticide, Fungicide, and Rodenticide Act
  function, 3–4
  reregistration process, 4–6
Fenchlorazole-ethyl, use as synergist, 50
Fenoxycarb, insecticidal activity, 150
Fenpiclonil, activity and structure, 319,320*t*
Fentrifanil, structure, 227
Fermentation, role in commercialization of bioherbicides, 84–85
Ferulic acid, phytotoxicity, 112
Flufenoxuron
  insecticidal activity, 146–147*t*
  structure, 145*f*,146
6-Fluoro-5-enolpyruvylshikimate-3-phosphate
  function, 22–24
  structure, 22,23*f*
Foil development, 263,265
Foreign genes, insecticidal effect on recombinant baculoviruses, 287,289–290
Forests, use of baculoviruses for pest control, 249–250
Fosetyl aluminum, use as fungicide, 334–335
Fumonisin B_1, phytotoxicity, 118
Fungal-based bioherbicides
  examples, 88,89*t*,91*t*,92
  sicklepod results, 92,93*f*
  specificity, 88
  velvetleaf results, 89,90*f*
Fungicides
  advantages, 333
  application areas, 332
  environmental considerations, 336–338
  history, 333–335
  human exposure, 335–336
  industrial perspective, 338–340

G

Genetic engineering, plant science effect, 269
Genetic manipulation, alteration of mycoherbicide host range, 101–108
Genetic modification of endemic plant pathogens, use in weed control, 11
Genetic validation, targets for herbicide design, 19–21
Geraniol, nematicidal activity and structure, 306,307*f*
Glaucarubolone, nematicidal activity and structure, 310*f*,311
Glufosinate
  advantages for generation of tolerant crops, 43
  phytotoxicity, 43,115
  properties for ideal chemical weed control, 42*t*,43
  structure, 115,116*f*
  synergism with bacteria, 96,98*f*
Glufosinate-tolerant crops
  field use, 44,45*t*,46
  tolerance acquisition mechanisms, 43–44

Glutamine synthetase, inhibition, 27,28*f*
Glutathione, oxidant detoxification pathway suppression, 55
Glutathione transferases, inhibition by synergists, 51,52*t*
Glyceollin, nematicidal activity and structure, 308,309*f*
Glycinoeclepin A, nematicidal activity and structure, 310*f*,311
Glycosyl transferases, inhibition by synergists, 56
Gossypol, nematicidal activity and structure, 308,309*f*
Granulosis virus, description, 244
Ground-state analogues
  examples, 22,23*f*,24
  use as enzyme inhibitors, 22
Groundwater contamination, public concern, 8–9
Group-specific reagents, 21
Growth, pesticide use, 2–3
Gypsy moth, control with baculoviruses, 249–250

H

Haber–Weiss reactions, description, 55
Halliwell–Asada pathway, suppression by copper chelator–synergists, 56
Halliwell–Asada pathway enzymes, resistance to herbicides, 55–56
Halogenated benzoic acid herbicides, phytotoxicity, 112
Heavy-metal ions, use as fungicides, 333
Helicoverpa virescens, control with baculoviruses, 248
Helicoverpa zea, control with baculoviruses, 248
Heliothis, control with baculoviruses, 248
Heptadeca-1,9-diene-4,6-diyne-3,8-diol, nematicidal activity and structure, 303,305*f*
Herbicide(s)
  crop tolerance, 7
  development from natural phytotoxins, 110–119
  interest in alternatives, 101
  soil and water contamination, 8–9
  target-site-directed design, 16–33
Herbicide design, effect of understanding of weed and crop biochemistry, 7–8
Herbicide resistance, influencing factors, 9–10
Herbicide-resistant crops, 11
Herbicide target site
  δ-aminolevulinic acid dehydratase, 73*f*
  protoporphyrinogen oxidase, 68,69–71*f*
Hessian fly, control with resistant gene, 268
History, fungicides, 333–335
HOE 704
  discovery, 19
  use as enzyme inhibitor, 27,28*f*
Honeybees, ecological effects of methoprene, 156–157
Host plant resistance
  definition, 267
  developmental process, 268–269
Host range of mycoherbicides, alteration by genetic manipulation, 101–108
Human exposure, fungicides, 335–336
Hymenopteran insects, prey incapacitation, 233
Hypericin, phytotoxicity and structure, 112,113*f*

I

Imazaquin
  structure, 29,31*f*
  use as enzyme inhibitor, 31–32
Imidacloprid
  activity, 185–187
  broad spectrum of activity and broad crop applicability, 184,185–188*t*
  comparison to nitromethylenes, 184
  development, 183
  ecological effect, 191,193
  environmental fate, 191
  favorable properties for applicator, consumer, and environment, 188–192
  formulations tested, 185,188*t*
  mammalian toxicity, 188,189*t*,190
  margins of safety, 190–191,192*t*
  potential for resistance, 193–195
  structure, 184
  tolerance assessment analysis, 190*t*
  tool for integrated pest management, 195–197
  tool for resistance management, 193–194,195*t*

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

In vitro activity, translation to in vivo efficacy, 32
Indigenous fungi, efficacy, 6
Inducer-type modulators, mechanism of action, 65–66
Industrial perspective for fungicides
  low use rates, 338
  mode of action, 338–339
  mutagenicity parameters, 339–340
  similarity to known hazardous materials, 339
  stability in soil and water, 339
  toxicity to nontarget species, 339
Inhibitors, noncompetitive blocker site on γ-aminobutyric acid receptors in mammals, 132–133
Insect(s)
  noncompetitive blocker site on γ-aminobutyric acid receptors, 134*t*,135*f*,136*t*,137*f*
  use in weed control, 11
Insect control in transgenic plants, *Bacillus thuringiensis* endotoxin, 271
Insect growth regulators
  chitin synthesis inhibitors, 144–149
  degradation in environment, 160–165
  groups, 144
  juvenile hormone analogues, 148–153
  mode of action, 144
  nontarget organism effect, 153–157
Insect pest control, use for peptides, 284–285
Insect viruses for pest control, 281–282
Insecticidal activity
  chitin synthesis inhibitors, 146–147*t*
  juvenile hormone analogues, 150*t*,152*t*
Insecticidal crystal proteins
  Bacillus thuringiensis, 261,271–272
  families, 261
Insecticide(s), soil and water contamination, 8
Insecticide action, primary targets, 126
Insecticide binding sites on γ-aminobutyric acid receptors of insects and mammals
  avermectin binding site, 136,138,139*f*,140
  binding sites, 128*f*,129
  future research, 140
  noncompetitive blocker(s), 129,130–131*f*,132
  noncompetitive blocker site in insects, 134*t*,135*f*,136*t*,137*f*
Insecticide binding sites on γ-aminobutyric acid receptors of insects and mammals—*Continued*
  noncompetitive blocker sites in mammals, 132–133,135*f*
  study system, 126–127
Insecticide lures, use of semiochemicals, 207–208
Insecticide resistance, monitoring with semiochemicals, 202–203
Integrated crop management, definition, 39
Integrated pest management
  objectives, 316
  practices, 2
  use of imidacloprid, 195–197
  use of semiochemicals, 199–208
Isonicotinic acid derivatives, structure and systemic acquired resistance, 321
N-Isopropyloxalylhydroxamate, use as enzyme inhibitor, 27,28*f*
Ivermectin
  application, 169
  structure, 171,172*f*

J

Juvenile hormone(s)
  functions, 148,150
  structures, 148,149*f*
Juvenile hormone analogues
  insecticidal activity, 150*t*,152*t*
  mode of action, 152–153
  structures, 150,151*f*
Juvenile hormone esterase, insecticidal activity effect, 289

K

Ketol acid reductoisomerase
  inhibition, 27,28*f*
  inhibitors, 19
Kinins, irreversible depletion of cholinergic nerve endings, 234–235,236*f*
Klaineanone, nematicidal activity and structure, 310*f*,311

L

cis-Lachnophyllum and *trans*-lachnophyllum esters, nematicidal activity and structures, 303–306

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Lepidopterous insects, *Bacillus thuringiensis* gene effect, 276–278
Limonene, nematicidal activity and structure, 306,307*f*
Linoleic acids, nematicidal activity and structures, 306,307*f*
Lipids, nematicidal activity and structures, 306,307*f*

M

Maculosin, phytotoxicity and structure, 116*f*,117
Mammals, noncompetitive blocker site on γ-aminobutyric acid receptors, 132–133,135*f*
Mass trapping of pests, use of semiochemicals, 206–207
Menadione, monooxygenase inhibition, 52–53,54*t*
Menthol, nematicidal activity and structure, 306,307*f*
Metabolic synergists
  amidase inhibition, 56
  biocontrol agent synergism, 57
  definition, 49
  esterase inhibition, 56
  glutathione transferase inhibition, 51,52*t*
  glycosyl transferase inhibition, 56
  limitations, 50–51
  monooxygenase inhibition, 52–53,54*t*
  oxidant detoxification pathway suppression, 54–56
  research value, 49–50
  role in lowering pesticide use levels, 49
  selectivity, 50
  target site resistances, 57
Methoprene
  ecological effects, 154–157
  insecticidal activity, 150
Methyl [(4-aminophenyl)sulfonyl]carbamate, function and structure, 22,23*f*
Methyl 2-*cis*,8-*cis*-deca-2,8-diene-4,6-diynoate, nematicidal activity and structure, 303,304*f*
Metyrapone, monooxygenase inhibition, 52–53,54*t*
Microbial degradation, prevention of buildup of fungicide, 337
Microbial metabolite derivatives, use for disease control, 316–321
Microbial phytotoxins with herbicidal potential
  development strategies, 114–115
  examples, 115,117–118
  factors affecting discovery and development, 118–119
  structures, 115,116*f*
Milbemycins
  applications, 169,171
  discovery, 169
  structures, 169–172
Mite toxin, insecticidal activity effect, 290
Mode of action, chitin synthesis inhibitors, 147–148,149*f*
Modulators
  herbicidal synergism with δ-aminolevulinic acid, 64,65*t*
  mechanism of action, 65–67
Monitoring with semiochemicals, types, 200–203
Monocrotaline, nematicidal activity and structure, 302*f*,303
Monooxygenases, inhibition by synergists, 52–53,54*t*
Moxidectin, applications and structure, 171,172*f*
Multiple nucleocapsids per envelope, description, 244–245
Mycoherbicide host-range alteration by genetic manipulation
  Sclerotinia sclerotiorum model system, 102–106
  zip code system for auxotrophy, 106–107,108*t*
Myristic acid, nematicidal activity and structure, 306,307*f*

N

Natural phytotoxins as herbicides
  factors affecting discovery and development, 118–119
  history of development, 110–111
  microbial phytotoxins, 114–118
  plant-derived phytotoxins, 111–112,113*f*,114

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Natural products
  direct use in crop protection, 317*t*
  history of use for pest control, 110
  opportunities, 316–320
  phenylpyrrole fungicides, 319*f*,320*t*
  pyrrolnitrin, 318
  systemic acquired resistance, 321
  use as agricultural antibiotics, 317,318*t*
Naturally occurring nematicides
  acetylenes, 303,304–305*f*,306
  alkaloids, 301,302*f*,303
  fatty acids and lipids, 306,307*f*
  future prospects, 312
  other compounds, 308,310*f*,311
  phenolic compounds, 306,308,309*f*
  polythienyls, 301*f*
  postinfectional compounds, 308,309*f*
  terpenoids, 306,307*f*
Nematicides
  development of environmentally safe compounds, 300–301
  disadvantages, 300
  naturally occurring, *See* Naturally occurring nematicides
Neuropeptides, use in construction of recombinant baculoviruses, 285–286
Neurotoxic insecticides, arthropod derived, *See* Arthropod-derived neurotoxic insecticides
Nitrodiphenyl ethers, oxidant detoxification pathway suppression, 54
Nitrogen-derivatized pyrroles, insecticidal activity, 223,226*f*,227*t*
Nitroguanidine insecticide, imidacloprid, 183–197
Nitromethylenes, comparison to imidacloprid, 184
Noncompetitive blocker(s), insecticide binding sites on γ-aminobutyric acid receptors of insects and mammals, 129,130–131*f*,132*t*
Noncompetitive blocker site on γ-aminobutyric acid receptors in insects
  comparison to that of mammals, 134*t*,136*t*,137*f*
  inhibitors, 134,135*f*
  resistance mechanism, 136,137*f*
Noncompetitive blocker site on γ-aminobutyric acid receptors in mammals
  affinity probes, 133
  binding kinetics, 133,135*f*
Noncompetitive blocker site on γ-aminobutyric acid receptors in mammals—*Continued*
  comparison to that of insects, 134*t*,136*t*,137*f*
  inhibitors, 132–133
Nonconventional chemical pest controls, approaches, 10
Nonrecombinant approaches, development of improved *Bacillus thuringiensis* products, 263
Nonscorpion venom toxins, use in construction of recombinant baculoviruses, 287
Nontarget effects of fungicides, minimization, 332–340
Nuclear polyhedrosis virus
  description, 244
  use as insecticide, 281
Nun moth, control with viruses, 239–240
Nutrient competition, use in biocontrol, 328,329*t*

O

Odoracin, nematicidal activity and structure, 310*f*,311
Odoratrin, nematicidal activity and structure, 310*f*,311
Orchard crops, use of baculoviruses for pest control, 248
Organochlorine pesticides, environmental effects, 3
Organotins, use as fungicides, 333
Ornithine transcarbamoylase, inhibition, 29,30*f*
Oxidant detoxification pathway, suppression by synergists, 54–56

P

Paclobutrazol, monooxygenase inhibition, 52–53,54*t*
Palmitic acid, nematicidal activity and structure, 306,307*f*
Particle bombardment process, production of transgenic cotton plants, 270
Pathogen growth, retardation with biocontrol strategies, 324–325
Pathogen inoculum, destruction with biocontrol strategies, 325

Peptides, use for control of insect pests, 284–285
Pest control
  importance of discovery strategies, 219
  insect growth regulators, 144–165
Pest control approaches
  contamination concerns, 8–9
  selectivity, 6–8
Pest control chemicals, growth, 2–3
Pest control with enhanced toxicological and environmental safety, challenges, 1–12
Pest management
  future directions, 11–12
  impact of biotechnological methods, 10–11
Pest resistance, influencing factors, 9–10
Pest-resistant cultivars, advantages and examples, 267–268
Pesticide(s)
  advantages, 2
  environmental effects, 3
  factors affecting growth, 2–3
  Federal Insecticide, Fungicide, and Rodenticide Act, 3–6
  human exposure routes, 6
  public policy, 6
  use of synergists to reduce use rates, 48–57
Pesticide chemicals, market value, 1
Pesticide use, growth, 2–3
Phaseolotoxin, phytotoxicity, 117
Phenolic compounds
  nematicidal activity, 306,308
  structures, 308,309*f*
Phenopylate analogues, quantitative structure–activity relationships, 72
Phenyl saligenin cyclic phosphonate, esterase inhibition, 56
Phenylalanine ammonia lyase, inhibition, 21
Phenylamides, use as fungicides, 335
1-Phenylhepta-1,3,5-triyne, nematicidal activity and structure, 303,304*f*
2-Phenyl-5-(1′-propynyl)thiophene, nematicidal activity and structure, 303,304*f*
Phenylpyrrole fungicides, activity, 319
N-Phenyltetrahydrophthalimides, quantitative structure–activity relationships, 72
Phenylureas, oxidant detoxification pathway suppression, 54
Philanthotoxin 4.3.3, structure, 234,236*f*
Phosalacine, phytotoxicity and structure, 115,116*f*
L-Phosphinothricin, description, 43
Phosphinothricin acetyltransferase, transformation of crops to glufosinate-tolerant crops, 44
Phosphinothricin acetyltransferase gene, use in gene transfer, 44
N-(Phosphonoacetyl)-L-aspartate, use as enzyme inhibitor, 29,30*f*
N-(Phosphonoacetyl)-L-ornithine, use as enzyme inhibitor, 29,30*f*
Phototoxicity, measurement from chlorophyll content, 62
Phthalimides, use as fungicides, 333
Physostigmine, nematicidal activity and structure, 301–303
Phytoparasitic nematodes, disadvantages of control chemicals, 300
Phytotoxins
  microbial, *See* Microbial phytotoxins with herbicidal potential
  natural, *See* Natural phytotoxins as herbicides
  plant derived, *See* Plant-derived phytotoxins with herbicidal potential
Picrotoxinin, noncompetitive blocker of γ-aminobutyric acid receptor binding sites, 129,130*f*
Pink bollworm, *Bacillus thuringiensis* gene effect, 278
Piperonyl butoxide, monooxygenase inhibition, 52–53,54*t*
Plant breeding, use in integrated crop management, 39
Plant defenses, use for disease control, 316–321
Plant-derived phytotoxins with herbicidal potential
  development strategies, 114
  examples, 111–112,114
  factors affecting discovery and development, 118–119
  structures, 112,113*f*
Plant protection chemicals, annual application, 38*t*
Pogo strategy, description, 107
Polyamine toxins, reversible noncompetitive block of synaptic transmission, 234,236*f*
Polychlorocycloalkanes, noncompetitive blockers of γ-aminobutyric acid receptor binding site, 129,130*f*,132

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Polythienyls, nematicidal activity and structures, 301
Poneratoxin, induction of interconversion between two gating modes of sodium channels, 235
Porphobilinogen deaminase as herbicide target site, inhibition, 73
Porphyric herbicides
  δ-aminolevulinic acid, 64–68
  function, 63–64
Porphyrin pathway, reactions, 63*f*
Porphyrin pathway as herbicide target site
  δ-aminolevulinic acid as porphyric herbicide, 64–68
  design, 75–76
  enzymes, 72,73*f*
  importance, 62–63
  protoporphyrinogen oxidase, 68,69–71*f*
  quantitative herbicide structure–activity relationships, 72
  relationship of substrate macrocycle and backbone structure of inhibitor, 73,74*f*,75
Postinfectional compounds, nematicidal activity and structures, 308,309*f*
Poxviridae, applications, 240–241
Preemergence herbicides, use as insurance policy against pest attack, 48
Propachlor, structure, 22*f*
Propargyl glycine, use as enzyme inhibitors, 25,26*f*
Propiconazole, monooxygenase inhibition, 52–53,54*t*
Protogen
  molecular similarities of inhibitors to protoporphyrinogen oxidase inhibitors, 70*f*,71
  proposed oxidation pathway, 74*f*,75
  structure, 71*f*
Protoporphyrin IX, phytotoxicity and structure, 112,113*f*,114
Protoporphyrinogen oxidase as herbicide target site
  competitive inhibition, 69,71
  inhibition by herbicides, 68–69
  molecular similarities of inhibitors to Protogen inhibitors, 70–71*f*
  proposed oxidation pathway, 74*f*,75
  quantitative structure–activity relationships, 72
  reasons for advantages, 75–76
Protoporphyrinogen oxidase inhibiting herbicides, synergism with δ-aminolevulinic acid, 67–68
Protox, *See* Protoporphyrinogen oxidase
Pyridazinones, oxidant detoxification pathway suppression, 54
Pyriproxyfen
  aerobic soil metabolism, 160
  bioaccumulation and metabolism in aquatic organisms, 163,164*f*
  bioavailability, 163,165*t*
  Daphnia pulex reproduction effect, 157,158–159*f*,160
  decline in natural ponds, 161
  degradation in water–sediment system, 160,161*t*,162*f*
  efficacy, 150,152*t*
  insecticidal activity, 150
  photodegradation, 160
Pyrocatechol, nematicidal activity and structure, 308,309*f*
Pyrroles
  AC 303,630, 228,229*t*,230–231*f*
  insecticidal activity, 223–231
  mode of action, 227,228*t*
  structure–activity relationship, 227
  synthesis, 220,221–222*f*,224*f*
Pyrrolnitrin, 318–320

Q

Quercetin, nematicidal activity and structure, 308,309*f*
Quinones, use as fungicides, 333

R

Rape seed
  rate of glufosinate required for weed control for tolerant crops, 45*t*,46
  weed control systems, 40–41*t*
Reaction intermediate analogues
  structures, 27,28*f*,29,30*f*
  use as enzyme inhibitors, 25–30
Recombinant approaches, development of improved *Bacillus thuringiensis* products, 264–266
Recombinant baculoviruses carrying foreign genes, insecticidal activity effect, 287

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Recombinant DNA technology, plant science effect, 269
Redhead pine sawfly, control with baculoviruses, 249
Reliability, pest control approaches, 6
Reoviridae, applications, 241,244
Repellents, use of semiochemicals, 208
Reregistration, process, 4–6
Resistance management, use of imidacloprid, 193–194
Resistance mechanisms, *Bacillus thuringiensis* gene use in transgenic cotton to control lepidopterous insects, 273–274
Resistance to pesticides, influencing factors, 9–10
Rhizobacteria, synergism with chemical herbicides, 92,94–99
Rhizosphere, protection by using biocontrol strategies, 324
Ribulose-1,5-bisphosphate carboxylase and oxygenase, 18
Rice cultivars, control of brown planthopper, 268
Rishitin, nematicidal activity and structure, 308,309*f*
Rubisco, use as target for herbicide design, 18

S

Safety, challenge of pest control, 1–12
Saligenin cyclic phosphates, glutathione transferase inhibition, 51,52*t*
Salt-marsh caterpillar, *Bacillus thuringiensis* gene effect, 278
Sanguinarine, nematicidal activity and structure, 302*f*,303
Sclerotinia sclerotiorum model system
  mutant(s), 102,104*t*
  mutant toxicity, 104,105*f*,106
  strain improvement via mating, 106
  weed hosts, 102,103*t*
Scorpion venom toxins
  insecticidal activity effect, 290
  use in construction of recombinant baculoviruses, 286–287,288*f*
Search for new chemicals, influencing factors, 316–317
Selection, targets for herbicide design, 17–18
Selectivity
  metabolic synergists, 50
  pest control approaches, 7–8
Semiochemicals
  definition, 199
  development, 6
  uses, 199–200
  integrated pest management programs
    advantages for monitoring, 200
    antifeedants, 208
    behavioral disruption in pests, 205–206
    blend composition, 204–205
    containment and concentration of pests, 206
    estimation of density of pests, 202
    examples, 200
    exotic pest monitoring, 201
    increased parasitoid and predator searching, 208
    insecticide lures, 207–208
    insecticide resistance monitoring, 202–203
    lure design, 203
    mass trapping of pests, 206–207
    monitoring of dispersal and extent of distribution of pests, 201–202
    monitoring of time of first appearance of pests, 200–201
    repellents, 208
    technical considerations, 203–205
    trap design, 203–204
Semisynthetic avermectins, yields, 176*t*
Single nucleocapsid per envelope, description, 244–245
Sodium channels, interconversion between two gating modes induced by poneratoxin, 235,236*f*
Soil, photodegradation of pyriproxyfen, 160
Soil contamination concerns, pest control approaches, 8–9
Soilborne plant pathogens, strategies and techniques for improving biocontrol, 323–329
Soybeans, use of baculoviruses for pest control, 249
Specificity, pest control approaches, 6–7
Spermosphere, protection by using biocontrol strategies, 324
6,5-Spiroketal avermectins, 176–179
Spotted knapweed, amino acid profile, 107,108*t*

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Stabilization, role in commercialization of bioherbicides, 84–85
Sterol biosynthesis inhibitors, use as fungicides, 334
24,25-Substituted avermectins, activity, 176,178*t*
Sugar beet
  rate of glufosinate required for weed control for tolerant crops, 45*t*,46
  weed control systems, 40*t*,41,42*t*
Suicide substrates, use as enzyme inhibitors, 25,26*f*
Sulfonylureas, advantages, 39
Sulfosate, synergism with bacteria, 94–97,99
Sulfur, use as fungicide, 333
Superoxide dismutase, oxidant detoxification pathway suppression, 55
Surface water contamination, public concern, 8–9
Synaptic transmission, reversible noncompetitive block by polyamine toxins, 234,236*f*
Synergist(s)
  definition, 49
  function, 48–49
Synergistic mixture, definition, 49
Synthetic chemical pesticides
  description, 7
  development of alternatives, 258
Synthetic herbicides, factors affecting use, 79
Systemic acquired resistance, 321
Systemic fungicides, 334

T

Tabtoxin, phytotoxicity, 117
Tabtoxinine-β-lactam, phytotoxicity, 117
Target selection for herbicide design, criteria, 17–18
Target site directed herbicide design
  enzyme inhibitor design, 21–32
  processes, 16–17
  target selection, 17–18
  target validation, 18–21
  translation of in vitro activity to in vivo efficacy, 32–33
Target site resistances, synergist design, 57
Target validation for herbicide design
  chemical validation, 18–19
  genetic validation, 19–21
  importance, 18
Teflubenzuron, insecticidal activity and structure, 145*f*,146
Tentoxin, 116–118
Terpenoids
  nematicidal activity, 306
  phytotoxicity, 114
  structures, 306,307*f*
Terrestrial environment
  ecological effects of diflubenzuron, 154
  ecological effects of methoprene, 156
α-Terthienyl
  nematicidal activity, 301
  phytotoxicity, 112
  structure, 112,113*f*,301*f*
Tetcyclacis, monooxygenase inhibition, 52–53,54*t*
Thiram, use as fungicide, 333
Tobacco budworm larvae, *Bacillus thuringiensis* gene effect, 277–278
α-Tocopherol, oxidant detoxification pathway suppression, 55
Tolerance setting, process, 4
Toxicity, pest control approaches, 8
Toxicological safety, challenge of pest control, 1–12
Toyocamycin, phytotoxicity, 115,117
Traits, use in biocontrol, 328–329
Transgenic cotton plants
  Bacillus thuringiensis gene management strategies, 274–276
  production systems, 270
Transgenic plant species, development, 6
Triazines, oxidant detoxification pathway suppression, 54
Triazolopyrimidine sulfonamide, structure, 29,31*f*
S,S,S-Tributyl phosphorotrithioate, esterase inhibition, 56
Tricyclazole, use as fungicide, 335
3-*cis*-Trideca-, 11-*trans*-trideca-, and 3-*trans*, 11-*trans*-trideca-1,3,11-trien-5,7,9-triynes, nematicidal activity and structure, 303,304*f*
Tridec-1-ene-3,5,7,9,11-pentayne, nematicidal activity and structure, 303,304*f*
Tridiphane
  glutathione transferase inhibition, 51,52*t*
  monooxygenase inhibition, 52–53,54*t*
2,6,7-Trioxabicyclo[2.2.2]octanes, noncompetitive blockers of γ-aminobutyric acid receptor binding site, 131,132*t*

In Pest Control with Enhanced Environmental Safety; Duke, S., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1993.

U

Uracils, oxidant detoxification pathway suppression, 54
Use rates, reduction by use of synergists, 48–57

V

Validation, targets for herbicide design, 18–21
Velvetbean caterpillar, control with baculoviruses, 249
Venom toxins, use in construction of recombinant baculoviruses, 286–287,288*f*
Vespulakinins, structures, 234,236*f*
4-Vinyl reductase as herbicide target site, inhibition, 73
Viruses, history of pest control use, 239–240
Viruses for pest control
  advantages, 245
  baculovirus applications, 244,246–250
  baculovirus characterization, 244–245
  criteria, 240
  developmental restraints, 250–254
  families used, 240,241*t*
  host specificity, 245
  inclusions of infectious virions in protein crystal, 240,242–243*f*
  lack of residue problems, 245
  patent protection, 254
  Poxviridae applications, 240–241
  production, 252–254
Viruses for pest control—*Continued*
  Reoviridae applications, 241,244
  slow action, 251–252
  specificity, 250–251
Vulgamycin, phytotoxicity and structure, 115,116*f*

W

Water, photodegradation of pyriproxyfen, 160
Water contamination concerns, pest control approaches, 8–9
Weed biocontrol agents–chemical herbicide synergism
  bacteria-based bioherbicides, 92,94–99
  fungal-based bioherbicides, 88–93
Weed control
  methods, 101
  systems, 40–43

X

X-tend bioherbicide system
  development, 92,94
  field trials, 96,97–99*f*
  preparation, 94,95*f*,96
Xanthomonas campestris pv. *poannua*, use as bioherbicide, 82–83

Z

Zip code system, auxotrophy, 106–107,108*t*

Downloaded by 89.163.35.42 on October 26, 2012 | http://pubs.acs.org
Publication Date: March 12, 1993 | doi: 10.1021/bk-1993-0524.ix002

Production: Donna Lucas
Indexing: Deborah H. Steiner
Acquisition: Anne Wilson

Printed and bound bv Maple Press. York. PA